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(54) Title: GENE THERAPY FOR METABOLITE DISORDERS

(57) Abstract

The present invention is directed to recombinant mammalian stem cells which contain a heterologous gene which encodes an enzyme that alters or degrades a metabolite, and the use of such cells in the treatment or prevention of a mammalian disease or disorder resulting from an increased concentration or accumulation of a metabolite. The invention also relates to compositions useful for the production of such recombinant mammalian stem cells. The recombinant stem cells or their progeny are introduced into or onto the host, where they proliferate to produce stem cell progeny that express the heterologous enzyme capable of altering or degrading the metabolite in vivo. In a specific embodiment, a heterologous gene encoding an enzyme capable of metabolizing oxalate can be expressed in recombinant stem cells in order to treat or prevent kidney stone formation. In another embodiment, a heterologous enzyme which alters or degrades uric acid can be expressed, in order to treat or prevent kidney stone formation or gout.

GENE THERAPY FOR METABOLITE DISORDERS

1.   INTRODUCTION

The present invention is directed to recombinant mammalian stem cells expressing heterologous genes and the use of such cells in the treatment or prevention of a disorder resulting from increased concentration or accumulation of a metabolite. The invention provides for the generation of stem cell progeny expressing the recombinant gene product which alters or degrades the desired metabolite in vivo. In a particular embodiment of the present invention, a gene encoding an enzyme capable of metabolizing oxalate may be expressed in the stem cell progeny of the hematopoietic system, of the skin, or of the lining of the gut, in order to treat or prevent oxalosis and kidney stone formation. In another embodiment of the invention, a gene encoding an enzyme capable of metabolizing uric acid may be expressed by recombinant human stem cells to treat or prevent gout or kidney stones. In a different embodiment of the invention, immunoglobulin genes may be expressed in recombinant epithelial stem cell progeny.

2.   BACKGROUND OF THE INVENTION

2.1.   OXALATE METABOLISM

Three mechanisms for oxalate catabolism are known: oxidation, decarboxylation, and activation followed by decarboxylation (Hodgkinson, A., 1977, Oxalic Acid in Biology and Medicine, Academic Press, pp. 119-124). Oxalate oxidases are enzymes that are found in mosses, higher plants, and possibly fungi which catalyze the oxidation of oxalate to hydrogen peroxide plus carbon dioxide: $(COOH)_2 + O_2 \rightarrow 2CO_2 + H_2O_2$. Oxalate decarboxylases are enzymes which produce CO_2 and formate as products of oxalate degradation. An O_2-dependent oxalate decarboxylase found in fungi catalyzes the decarboxylation

of oxalic acid to yield stoichiometric quantities of formic acid and CO_2: $(COOH)_2 \rightarrow CO_2 + HCOOH$. Varieties of both aerobic and anaerobic bacteria can also degrade oxalic acid. An activation and decarboxylation mechanism is used

5   for degradation of oxalate in Pseudomonas oxaliticus and other bacteria, comprising the following steps (Hodgkinson, A., supra, p. 124):

oxalate + succinyl-CoA $\rightarrow$ oxalyl-CoA + succinate

oxalyl-CoA $\xrightarrow{TPP}$ formyl-CoA $\pm$ CO_2

10   formyl-CoA + succinate $\rightarrow$ formate + succinyl-CoA

formyl-CoA + oxalate $\rightarrow$ formate + oxalyl-CoA
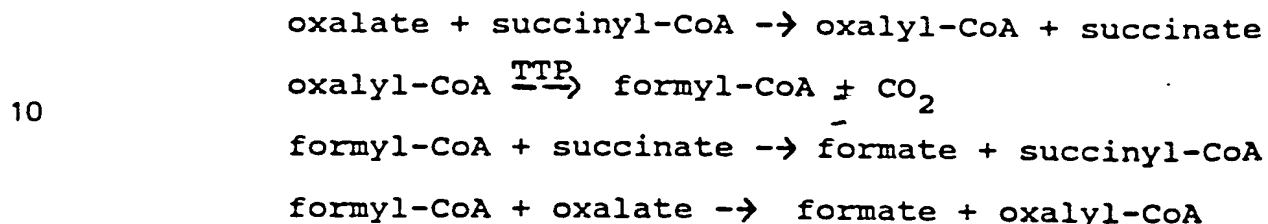
Oxalobacter formigenes is a recently described
15   oxalate-degrading anaerobic bacterium which inhabits the rumen of animals as well as the colon of man (Allison, M.J., 1985, Arch. Microbiol. 141:1-7). O. formigenes OxB is a strain that grows in media containing oxalate as the sole metabolic substrate. Other substrates do not appear
20   to support its growth. The degradation of oxalate catalyzed by the bacterial enzyme results in CO_2 and formic acid production (Allison, M.J., supra).

2.2.   OXALATE DISORDERS

25   There are no naturally occurring oxalate-degrading enzymes in vertebrates. Since humans do not metabolize oxalic acid, excess oxalic acid is not degraded to harmless products. Thus, excess ingestion of oxalic acid or its metabolic precursors can lead to acute oxalate toxicity.
30   Ingestion of ethylene glycol, diethylene glycol, xylitol, and excess ascorbic acid can lead through metabolic conversions to disorders of excess oxalate. Use of methoxyflurane as an anaesthetic can also lead to oxalosis. Aspergillosis, infection with an oxalate-producing fungus,
35   can lead to production and deposition of calcium oxalate.

Other causes of excess oxalic acid include renal failure,
intestinal disease, and primary hyperoxaluria, types I and
II (Hodgkinson, A., supra, pp. 217-241).

Excess oxalic acid can lead to various disorders.
5   These disorders include renal failure and oxalate
urolithiasis, an example of which is kidney stone
formation. Oxalate stones and other calculi, which are
comprised of crystals of calcium oxalate, can form in
urine, tissues, blood, etc. Acute oxalate toxicity can
10   cause local corrosive effects, systemic effects, and renal
failure. The possible systemic effects include
convulsions, shifts of the nervous system from acute mania
to coma, and death from cardiovascular collapse or central
nervous system depression (Id. at 217).

15   Oxalate toxicity can also cause livestock poisoning,
due to grazing on oxalate-rich pastures. Ingestion of
oxalate-rich plants such as Halogeton glomeratus, Bassia
hyssopifolia, Oxalis pes-caprae, and Setaria sphacelata, or
grains infected with the oxalate-producing fungi
20   Aspergillus niger, has been reported to cause oxalate
poisoning in sheep and cattle. Chronic poisoning is often
accompanied by appetite loss and renal impairment. Acute
toxicity can lead to tetany, coma, and death (Hodgkinson,
A., supra, pp. 220-222).

25
2.3. URIC ACID METABOLISM

In primates, Dalmatian dogs, birds, and some
reptiles, the end-product of purine degradation is uric
acid. In man, the purines adenine and guanine are
converted to xanthine, which is then oxidized by xanthine
30   oxidase to form uric acid: xanthine + H_2O + O_2 $\rightarrow$ uric acid
+ O_2^-. Superoxide dismutase converts the superoxide
radical (O_2^-) to hydrogen peroxide (Lehninger, A.L., 1975,
Biochemistry, 2d Ed., Worth Publishers, New York, pp. 740-
35   741).

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Enzymes which metabolize uric acid are
ubiquitous in mammals other than man or Dalmatian coach
hounds. In such ureotelic mammals, urate is reabsorbed in
the proximal convolution of the kidney and transported to
5   the liver where it is converted by hepatic uricase to
water-soluble allantoin. However, man lacks uricase, and
is thus genetically predisposed to uric acid urolithiasis
(Gutman, A.B., and Yu, T.-F., 1968, Am. J. Med. 45:756-
779).

10   A cDNA clone representing the porcine urate oxidase
gene has been isolated (Lee, C.C., et al., 1988, Science
239:1288-1291).

2.4.   URIC ACID DISORDERS

15   Uric acid in the blood is mainly present as a
monosodium salt. However, in some individuals, uric acid
precipitates out of solution, forming calculi of uric acid.
Excess uric acid in the serum causes the deposition of uric
acid in cartilaginous tissues, producing gout.
20   Hyperuricemia can also induce renal failure. Excess uric
acid in the urine and kidney can lead to uric acid
nephrolithiasis, which takes the form of kidney stones,
sand, and gravel, causing pain and damage to the kidney.
Uric acid kidney stones are composed of anhydrous uric
25   acid, which may be in conjunction with uric acid dihydrate,
and/or oxalates and phosphates (Gutman, A.B., and Yu, T.-
F., 1968, Am. J. Med. 45:756-779).
        Overproduction of uric acid leading to
hyperuricemia and uric acid nephrolithiasis has been shown
30   to be associated with inborn errors of metabolism which
occur in primary gout, Lesch-Nyhan syndrome, and glycogen
storage diseases, and with myeloproliferative and other
neoplastic disorders. Excess extrarenal water loss can
also lead to uric acid nephrolithiasis. Treatment with
35   uricosuric drugs, ingestion of excess purines and proteins,

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or defects in renal reabsorption of uric acid can all cause
hyperuricosuria and concomitant uric acid calculi
formation.   (Gutman, A.B. and Yu, T.-F., supra).

5   2.5.   GENE THERAPY

Gene therapy refers to the transfer and stable
insertion of new genetic information into cells for the
therapeutic treatment of diseases or disorders. The
foreign gene is transferred into a cell that proliferates
10   to spread the new gene throughout the cell population.
Thus stem cells, or pleuripotent progenitor cells, are
usually the target of gene transfer, since they are
proliferative cells that produce various progeny lineages
which will potentially express the foreign gene.

15   Although the development of high efficiency gene
transfer systems for hematopoietic progenitor cell
transformation will undoubtedly be useful in studying
hematopoietic cell differentiation, gene regulation, ways
to manipulate the immune response, and the like, the
20   ultimate goal will be gene therapy (Morrow, J.F., 1976,
Ann. N.Y. Acad. Sci. 265:13; Salzar, W., et al., 1981, in
Organization and Expression of Globin Genes, A.R. Liss,
Inc., New York, p. 313; Bernstein, A., 1985, in Genetic
Engineering: Principles and Methods, Plenum Press, New
25   York, p. 235; Dick, J.E., et al., 1986, Trends in Genetics
2:165). Reports on the development of viral vector systems
indicate a higher efficiency of transformation than DNA-
mediated gene transfer procedures (e.g., $CaPO_4$
precipitation and DEAE dextran) and show the capability of
30   integrating transferred genes stably in a wide variety of
cell types. Recombinant retrovirus vectors have been
widely used experimentally to transduce hematopoietic
progenitor cells (hematopoietic stem cells, HSCs). Genes
that have been successfully expressed in mice after
35   transfer by retrovirus vectors include human hypoxanthine

phosphoribosyl transferase (Miller, A., et al., 1984,
Science 255:630) and human β-globin (Dzierzak, E.A., et
al., 1988, Nature 331:35-41). Bacterial genes have also
been transferred into mammalian cells, in the form of
5 bacterial drug resistance gene transfers in experimental
models. The transformation of hematopoietic progenitor
cells to drug resistance by eukaryotic virus vectors, has
been accomplished with recombinant retrovirus-based vector
systems (Hock, R.A. and Miller, A.D., 1986, Nature
10 320:275-277; Joyner, A., et al., 1983, Nature 305:556-558;
Williams, D.A., et al., 1984, Nature 310:476-480; Dick,
J.E., et al., 1985, Cell 42:71-79); Keller, G., et al.,
1985, Nature 318:149-154; Eglitis, M., et al., 1985,
Science 230:1395-1398).

15   Adeno-associated virus (AAV) is a small,
helper-dependent human parvovirus consisting of three
structural genes, rep, lip and cap, which code for a
replication and two capsid proteins, respectively. A lytic
growth cycle for AAV requires co-infection with a helper
20 virus such as adenovirus or herpes simplex virus
(Atchinson, R.W., et al., 1965, Science 149:754-756;
Buller, R.M.L., et al., 1981, J. Virol. 40:241-247). In
the absence of helper virus, AAV stably integrates into the
host genome by recombination between the AAV terminal
25 repeats and host sequences, establishing a latent infection
(Berns, K.I., et al., 1982, in Virus Persistance, Mahy,
B.W.J., A.C. Mirson, and G.K. Darby, eds., Cambridge
University Press, New York, pp. 249-265; Cheung, A. K.-M.,
1980, J. Virol. 33:739-748). Subsequent co-infection of
30 latently infected cells with helper virus results in the
rescue of the AAV genome and a productive lytic cycle
(Hermonat, P.L., and Muzyczka, N., 1984, Proc. Natl. Acad.
Sci. U.S.A. 81:6466-6470; Hoggan, M.D., et al., in
Proceedings of the Fourth Lepetit Colloquium, North-Holland
35 Publishing Co., Amsterdam, pp. 243-249). Recently, AAV

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vectors have been used successfully to transduce mammalian
cell lines to neomycin resistance (Hermonat, P.L. and
Muzyczka, N., 1984, supra; Tratschin, J.-D., et al., 1985,
Mol. Cell. Biol. 5:3251).

5   Other viral vector systems that have been
investigated for use in gene transfer include papovaviruses
and vaccinia viruses (see Cline, M.J., 1985, Pharmac. Ther.
29:69-92).

          Other methods of gene transfer include
10 microinjection, electroporation, liposomes, chromosome
transfer, and transfection techniques (Cline, M.J., 1985,
supra). Salser et al. used a calcium-precipitation
transfection technique to transfer a methotrexate-resistant
dihydrofolate reductase (DHFR) or the herpes simplex virus
15 thymidine kinase gene, and a human globin gene into murine
HSCs. In vivo expression of the DHFR and thymidine kinase
genes in stem cell progeny was demonstrated (Salser, W., et
al., 1981, in Organization and Expression of Globin Genes,
Alan R. Liss, Inc., New York, pp. 313-334).

20   Gene therapy has also been investigated in murine
models with the goal of enzyme replacement therapy. Thus,
normal HSCs from a donor mouse have been used to
reconstitute the hematopoietic cell system of mice lacking
beta-glucuronidase (Yatziv, S., et al., 1982, J. Lab. Clin.
25 Med. 90:792-797). Since a native gene is being supplied,
no recombinant stem cells (or gene transfer techniques) are
used.

          As an alternative to gene therapy, the use of
erythrocytes as cellular carriers of therapeutic enzymes
30 was investigated by Sprandel (Sprandel, U., 1985, Biblthca
haemat. 51:714). In this study, the urate oxidase enzyme
itself was encapsulated in erythrocytes by dialysis.

35

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3. SUMMARY OF THE INVENTION

The present invention is directed to recombinant mammalian stem cells which express a heterologous gene which encodes an enzyme that alters or degrades a

5 metabolite (hereinafter termed metabolase), and to compositions useful for the production of such recombinant mammalian stem cells. The invention is also directed to the use of such cells in the treatment of a mammalian (including but not limited to human) disease or disorder

10 resulting from an increased concentration or accumulation of a metabolite. The invention provides for the treatment or prevention of such a disorder by the introduction into or onto the host of recombinant stem cells or their progeny, and the in vivo generation of stem cell progeny

15 expressing the recombinant metabolase gene product which alters or degrades the metabolite in vivo. In a particular embodiment of the present invention, a heterologous gene encoding an enzyme capable of metabolizing oxalate may be expressed in the stem cell progeny of the hematopoietic

20 system, of the skin, of the lining of the gut, or of embryonic heart muscle. Such stem cell progeny can be effective in treating or preventing disorders of excess oxalate such as kidney stones, renal failure, acute oxalic acid toxicity, and others. In another embodiment of the

25 invention, stem cell progeny may express a heterologous gene product capable of metabolizing uric acid, in order to treat or prevent such disorders as kidney stones, gout, and renal failure.

The present invention also relates to the use of

30 molecular modeling of the enzyme expressed by a cloned metabolase gene in order to produce synthetic chelators of the metabolite for therapeutic purposes.

The invention is also directed to recombinant stem cell progeny which express heterologous immunoglobulin

35 genes.

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3.1. DEFINITIONS

The following terms and abbreviations will have the meanings indicated:

5	AAV	=	adeno-associated vector.
	AAV/Oxy	=	a recombinant adeno-associated vector containing a bacterial-derived oxalase gene.
	K-Ox	=	potassium oxalate.
10	CFU	=	colony-forming unit. A cell which is capable of producing a colony of progeny cells in soft agar medium.
15	G/E/M CFU	=	granulocyte/erythrocyte/ macrophage colony forming unit. A colony forming unit capable of producing colonies composed of granulocyte, erythroid and macrophage progeny.
20	ESC	=	epithelial stem cell. A pleuripotent progenitor cell of the epithelial cell lineage.
25	HSC	=	hematopoietic stem cell. A pleuripotent progenitor cell of the hematopoietic cell lineage.
	Metabolase	=	an enzyme which catalyzes the alteration or degradation of a metabolite.
30	Oxalase	=	an enzyme which catalyzes the alteration or degradation of oxalic acid/oxalate.

35

Uricase	=	an enzyme which catalyzes the alteration or degradation of uric acid/urate.
Heterologous gene	=	a gene which is not present, or not expressed, in the designated host cell.
mAb	=	monoclonal antibody.
MLC	=	mixed leukocyte culture.
LPS	=	lipopolysaccharide.
GVH	=	graft versus host.
C'	=	complement.

The term "metabolite" as used herein is meant to include a compound which comprises an initial, as well as a subsequent, substrate of a metabolic pathway.

4. DESCRIPTION OF THE FIGURES

Figure 1. Physical organization of AAV recombinant genomes. Solid boxes indicate the position of the coding sequences for the rep, lip, and cap functions of AAV while the cross-hatched box represents a fragment of lambda-phage DNA inserted into the AAV genome. The interruption in the dl52-91 line indicates the position of the deletion in this mutant. A 1.8 kilobase pair insert containing the SV40 early promoter region (stippled box) and the bacterial neomycin resistance gene (striped box) was ligated into the AAV deletion mutant dl52-91 producing dl52-91/neo.

Figure 2. Morphology of a normal myeloid cell colony (A) and a G-418 resistant colony (B). Soft agar cultures were used to detect hematopoietic progenitor cells which were successfully transduced with the AAV/Neo vector. Colony forming units were grown in the presence of L cell

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conditioned medium. Normal colonies showed a slight spreading. G-418 resistant colonies appeared normal, although more compact, and contained several hundred cells within seven days after seeding.

Figure 3. Figure 3 represents a murine hematopoietic cell culture which was established using a modified Dexter cell culture system (Dexter et al., 1977, J. Cell. Physiol. 91:335), approximately three weeks after establishment. Good out-growth of an adherent cell population can be observed.

Figure 4. Figure 4 represents a long-term murine hematopoietic cell culture, established by reseeding cultures at the stage shown in Figure 3 with freshly prepared bone marrow cells added as a single cell suspension.

Figure 5. Cumulative survival curves of reconstituted and non-reconstituted lethally-irradiated hosts. A. (C57BL/10 x B10.BR/cd)F_1 and C57BL/6J mice non-reconstituted (▲——▲) or reconstituted with syngeneic bone marrow plus spleen cells (O——O). B. (C57BL/10 x B10.BR/cd)F_1 mice reconstituted with untreated (△——△) or newborn spleen cell pretreated (●——●) semi-allogeneic B10.BR/cd bone marrow plus spleen cells. C. C57BL/6J mice reconstituted with untreated (△——△) or newborn spleen cell pretreated (●——●) allogeneic B10.BR/cd bone marrow plus spleen cells. An asterisk indicates a time when an animal was killed for functional studies.

Figure 6. Histology of skin biopsies, liver and spleen sections. Non-reconstituted irradiation controls (a-c); long-term surviving allogeneic bone marrow plus newborn suppressor cell reconstituted animals (d-f); allogeneic bone marrow reconstituted animals undergoing GVH disease (g-i). Photographs of hematoxylin-eosin stained

sections were taken using 250 X magnification, except for
b,e and the inserts of c, f, and i which were taken using
100 X magnification.

Figure 7. In vitro immune responses of long-
term surviving, lethally-irradiated (C57BL/10 x
B10.BR/cd)F$_1$ mice reconstituted with B10.BR/cd bone marrow.
A. Spleen cell responses following stimulation with 25
ug/ml lipopolysaccharide (LPS) (O——O) or 8 ug/ml
Concanavalin A (Con A) (■——■). B. Mixed leukocyte
culture (MLC) responses of spleen cells against gamma-
irradiated B10.SAA48 (■——■), DBA/2J (▲——▲), CBA/J
(△——△), C57BL/10 (●——●) and B10.BR/cd (O——O)
spleen cells.

Figure 8. In vitro immune responses of long-
term surviving, lethally-irradiated C57BL/6J mice
reconstituted with B10.BR/cd bone marrow. A. Spleen cell
responses following stimulation with 25 ug/ml LPS (●——●)
or in unstimulated culture (●---●). B. MLC responses of
spleen cells against gamma-irradiated DBA/2J (▲——▲),
AKR/J (△——△) , B10.Q (■——■), C57BL/6J (●——●) and
CBA/H (O——O) spleen cells.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to recombinant
mammalian stem cells and their use in the treatment or
prevention of a disease or disorder due to an excess
concentration or accumulation of a metabolite. The
invention is also directed to compositions useful for the
production of such recombinant mammalian stem cells. The
recombinant stem cells of the present invention contain a
heterologous gene which encodes an enzyme capable of
altering or degrading the metabolite (termed hereinafter
"metabolase gene"). The recombinant stem cells or their
progeny are introduced into or onto the host, where they

proliferate to produce stem cell progeny that express the heterologous enzyme capable of altering or degrading the metabolite in vivo.

5.1. THE GENERATION OF RECOMBINANT STEM CELLS EXPRESSING A HETEROLOGOUS METABOLASE GENE

5.1.1. ISOLATION OF THE HETEROLOGOUS GENE

Any gene which encodes an enzyme capable of altering or degrading a disease- or disorder-causing metabolite may be used in accordance with the present invention. The gene may be derived from any heterologous organism, including bacteria or mammals, for expression in the desired mammalian stem cell, so long as it can be expressed under the control of a promoter in the mammalian stem cell and its cell progeny and provide for a functionally active nontoxic enzyme at the desired site of treatment.

Isolation of the metabolase gene involves the isolation of those DNA sequences which encode the peptide or protein with enzymatic activity. Such DNA may be obtained by standard procedures known in the art from cloned DNA, by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the organic acid-decomposing organism of interest. (See, for example, Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford U.K., Vol. I, II.) Whatever the source, the metabolase gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired metabolase. The DNA may be cleaved at specific sites using various restriction enzymes.

Alternatively, one may use DNase in the presence of
manganese to fragment the DNA, or the DNA can be physically
sheared, as for example, by sonication. The linear DNA
fragments can then be separated according to size by
5   standard techniques, including but limited to, agarose and
polyacrylamide gel electrophoresis and column
chromatography.

Once the DNA fragments are generated,
identification of the specific DNA fragment containing the
10   metabolase gene may be accomplished in a number of ways.
For example, if a small amount of the metabolase gene or
its specific RNA, or fragments thereof, is available and
can be labeled, the generated DNA fragments may be screened
by nucleic acid hybridization to the labeled probe. It is
15   also possible to identify the appropriate fragment by
restriction enzyme digestion(s) and comparison of fragment
sizes with those expected according to known restriction
maps if such are available. The gene can also be
identified by mRNA selection by nucleic acid hybridization
20   followed by in vitro translation. Other methods are
possible and within the scope of the present invention.

In a preferred embodiment, an oligonucleotide probe
capable of hybridizing to a DNA fragment containing the
oxalase gene may be obtained according to the following
25   procedure (see Section 8.1, infra): Oxalase protein
purified from a suitable bacteria, may be subjected to
amino acid sequencing to determine at least part of its
amino acid sequence. The derived amino acid sequence can
then be relied upon to deduce an appropriate DNA sequence
30   that is capable of hybridizing to the portion of the
oxalase gene encoding that amino acid sequence. An
oligonucleotide encoding the deduced DNA sequence can then
be synthesized and used to screen a bacterial genomic
library, for the detection and isolation of the oxalase
35   gene.

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The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or
5 modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives. Recombinant molecules can be introduced into host cells via
10 transformation, transfection, or infection.

In an alternative method, the metabolase gene fragment may be identified and isolated after insertion into a suitable cloning vector, in a "shot-gun" approach. Enrichment for the metabolase gene, for example, by size
15 fractionation, can be done before insertion into the cloning vector.

The metabolase gene is inserted into a cloning vector which is used to transform, transfect, or infect appropriate host cells so that many copies of the gene
20 sequences are generated. This can be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA
25 molecules may be enzymatically modified. Such modifications include producing blunt ends by digesting back single-stranded DNA termini, or by filling in the single-stranded termini so that the ends can be blunt-end ligated. Alternatively, any site desired may be produced
30 by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. For example, according to the DNA modification procedure of
35 Maniatis, sheared DNA is treated with a restriction

methylase (for example, M.EcoRI) and ligated to synthetic
DNA linkers for that enzyme. The DNA is then treated with
restriction endonuclease to cleave the terminal linkers
(but not the modified internal restriction sites) and

5   ligated to the appropriate vector arm. In an alternative
method, the cleaved vector and metabolase gene fragment may
be modified by homopolymeric tailing.

         Identification of the cloned metabolase gene can
be accomplished in a number of ways based on the properties

10   of DNA itself, or alternatively, on the physical, immunolo-
gical, or functional properties of its encoded enzyme. For
example, the DNA itself may be detected by plaque or colony
nucleic acid hybridization to labelled probes (Benton, W.
and Davis, R., 1977, Science 196:180; Grunstein, M. and

15   Hogness, D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961).
Alternatively, the presence of the metabolase gene may be
detected by an enzymatic assay based on the ability of the
expressed product to alter or degrade a specific substrate
metabolite. For example, a host cell expressing an oxalase

20   gene may be detected by metabolic breakdown of calcium
oxalate in the immediate environment, as described in
Section 7.1.1, infra. A recombinant host expressing a
uricase gene may be similarly selected by observing uric
acid hydrolysis within the immediate environment of the

25   recombinant host grown in a uric acid-containing medium.
If an antibody to the metabolase is available, the enzyme
may be identified by binding of labeled antibody to the
enzyme, in an ELISA (enzyme-linked immunosorbent assay)-
type method.

30   Once the metabolase DNA-containing clone has
been identified, it may be grown, harvested and its DNA
insert may be characterized as to its restriction sites by
various techniques known in the art (Maniatis, T., et al.,
Molecular Cloning, A Laboratory Manual, Cold Spring Harbor

35   Laboratory, New York).

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The sequence of the metabolase DNA insert can
then be determined. Methods by which this may be
accomplished include the Maxam-Gilbert procedure (Maxam,
A.M. and Gilbert, W., 1980, Meth. Enzymol. 65:499) or the
5 Sanger dideoxy chain termination procedure (Sanger, F., et
al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463). In a
specific embodiment employing the Sanger technique,
appropriate segments of the metabolase gene can be
preferably subcloned into M13 vectors (Messing, J., 1983,
10 Meth. Enzymol. 101:20) for optimal sequencing efficiency.
         Once the genetic structure of the metabolase
gene is known, it is possible to manipulate the structure
for optimal use in the present invention. For example,
promoter DNA may be ligated 5' of the metabolase coding
15 sequence, in addition to or replacement of the native
promoter, to provide for increased expression of the
enzyme. Cellular or tissue targeting sequences, if known,
may also be used to effect desired localization of the
metabolase in vivo. For example, DNA encoding a membrane
20 transport sequence can be ligated to the metabolase coding
region, so that the metabolase gene product will be
secreted. Many such manipulations are possible, and within
the scope of the present invention.

25   5.1.1.1. ISOLATION OF THE OXALASE GENE
         Genes which may be used in accordance with the
present invention for the treatment of disorders of oxalate
metabolism include those which encode oxalate oxidases and
oxalate decarboxylases. Oxalate decarboxylases yield
30 formic acid and carbon dioxide as products, and are thus
preferred for use in the present invention over oxalate
oxidases which yield the more irritant products in vivo of
hydrogen peroxide and carbon dioxide (Hodgkinson, A., 1977,
in Oxalic Acid in Biology and Medicine, Academic Press, pp.
35 119-120). The encoded enzyme should be one that has

optimal activity under the physiological conditions under
which it is desired to be employed. For example, those
oxalases which have significant activity at serum
temperature and pH levels would be suitable for a specific
5   embodiment of the present invention in which the
therapeutic oxalase activity is desired to treat increased
oxalate concentration within the blood.

Genes suitable for use in the present invention
may be isolated from any organism which expresses the
10 desired oxalase activity as long as the gene can be
functionally expressed in a human cell without toxic
effects. For example, oxalate oxidases can be isolated
from organisms including, but not limited to, mosses,
higher plants, and fungi. Oxalate decarboxylases can be
15 isolated from organisms including, but not limited to,
bacteria, plants, and fungi. In preferred embodiments, an
oxalate decarboxylase gene can be isolated from one of the
many oxalate-decomposing bacteria. Many such bacteria are
known, including, but not limited to, those listed in Table
20 I, <u>infra</u>, much of which is derived from Hodgkinson, A.,
1977, Oxalic Acid in Biology and Medicine, Academic Press,
pp. 122-124, which is incorporated by reference herein.

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TABLE I

BACTERIA CONTAINING OXALASE GENES WHICH MAY BE EXPRESSED IN RECOMBINANT STEM CELLS

Organism	Source	Reference
AEROBIC:		
Proactinomyces citreus	Soils, water and sheep rumen	Muller, 1950, Archiv fur Mikrobiol. 15: 137-148
Pseudomonas OD1	Garden soil	Jayasuriya, 1955, J. Gen. Microbiol. 12:419-428
Pseudomonas AM1	Aerial contaminant	Stocks and McCleskey, 1964, J. Bacteriol. 88:1065-1070
Pseudomonas AM2	Aerial contaminant	Blackmore and Quayle, 1970, Biochem. J., 118:53-59
Protaminobacter ruber	Aerial contaminant	Blackmore and Quayle, 1970, Biochem. J. 118:53-59
Pseudomonas extorquens	Aerial contaminant	Blackmore and Quayle, 1970, Biochem. J. 118:53-59
Pseudomonas M27	Garden soil	Anthony and Zatman, 1964, Biochem. J. 92:609-614
Pseudomonas RJ1	Soil	Mehta, 1973, Antonie van Leeuwenhoek J. Microbiol. Serol. 39:295-302

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Pseudomonas YOx	Chicken dung	Chandra and Shethna, 1975, Antonie van Leeuwenhoek J. Microbiol. Serol. 41:101-111
5 Pseudomonas MOx	Chicken dung	Chandra and Shethna, 1975, Antonie van Leeuwenhoek J. Microbiol. Serol. 41:101-111
10 Pseudomonas KOx	Chicken dung	Chandra and Shethna, 1975, Antonie van Leeuwenhoek J. Microbiol. Serol. 41:101-111
15 Alcaligenes LOx	Chicken dung	Chandra and Shethna, 1975, Antonie van Leeuwenhoek J. Microbiol. Serol., 41:101-111
Streptomyces spp. 20	-	Robbel and Kutzner, 1973, Die Naturwissenschaften 7:351-352
Thiobacillus novellus extorquen 25	Excreta of earthworm	Bassalik, 1913, Jahrbücher fur Wissenschaftliche Botanik 53:255-302
Vibrio oxaliticus	Soil	Bhat and Barker, 1948, J.Bacteriol. 55:359-368

ANAEROBIC:

30 Clostridium sp.	Donkey dung	Bhat, 1966, J. Scientific and Industrial Res., New Delhi 23:450-454

35

pseudomonad	soil	Jakoby and Bhat, 1958, Bact. Rev. 22:75-80
Pseudomonas oxalaticus		Quayle et al., 1961, Biochem. J. 78:225-236
Desulfovibrio vulgaris ssp. oxamicus 46:287-295	Mud enrichment cultures	Postgate, 1963, Arch. Mikrobiol.
Oxalobacter formigenes OxB	Human feces, sheep rumen, and pig cecum	Allison et al., 1985, Arch. Microbiol. 141:1-7

In a preferred embodiment of the present invention, the oxalate decarboxylase gene from O. formigenes OxB (American Type Culture Collection accession number 35274) can be expressed in recombinant stem cells. Since this gene encodes an enzyme which is active at serum temperature and pH, and is derived from a strain (O. formigenes) that is a normal inhabitant of the human gastrointestinal tract (Allison, M.J., et al., 1985, Arch. Microbiol. 141:1-7), it is likely to encode a nontoxic functional enzyme within a human host.

The term oxalase, as used herein, is meant to denote an enzyme which catalyzes the alteration or degradation of oxalic acid or oxalate, and thus the terms "oxalic acid" and "oxalate" are used interchangeably throughout the instant application unless clearly intended otherwise. In humans, the compound's physiological form is predominantly that of oxalate (which is probably the actual substrate for O. formigenes oxalase). Due to interconversion between the oxalic acid and oxalate forms, the metabolism of one form is effective for the treatment or prevention of a disorder caused by the other.

5.1.1.2. ISOLATION OF THE URICASE GENE

Since uricases are ubiquitous in mammals other than man and Dalmatian coach hounds, in a preferred embodiment the uricase gene can be isolated from mammals, particularly mammalian liver. In particular embodiments, the uricase gene can be isolated from bovine liver or porcine liver. Liver DNA can be prepared by standard procedures (Maniatis, T., et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

The term uricase, as used herein, is meant to denote an enzyme which catalyzes the alteration or degradation of uric acid or urate, and thus the terms "uric acid" and "urate" are used interchangeably throughout the instant application unless clearly intended otherwise. In humans, the compound's physiological form is predominantly that of urate (which is probably the actual substrate for mammalian uricase). Due to interconversion between the uric acid and urate forms, the metabolism of one form is effective for the treatment or prevention of a disorder caused by the other.

5.1.1.3. OTHER METABOLASE GENES

Other heterologous metabolase genes which may be expressed in recombinant mammalian stem cell progeny according to the present invention include those encoding an enzyme which alters or degrades a metabolite, such a metabolite including but not limited to a phenylketone, phenylpyruvic acid, phenylethylamine, a porphyrin or related molecule, delta-amino levulinic acid, testosterone, and cholesterol (See Section 5.3, infra). Such genes can be isolated from any suitable organism, as long as they encode a nontoxic functional product for humans. In a preferred embodiment, soil, fecal, or composte bacteria can be examined in order to identify those bacteria capable of

utilizing the desired molecule as a sole carbon and energy
source. For example, soil bacteria can be grown in media
containing cholesterol as the sole carbon and energy source
to determine if the bacteria contained therein have the

5 necessary enzymatic machinery with which to metabolize the
cholesterol. If the bacteria survive, their DNA can be
isolated, cloned, and selected for the desired metabolase
gene by methods such as described in Section 5.1.1 supra.
In an alternative embodiment, a metabolase gene such as

10 that encoding lecithin:cholesterol acyltransferase can be
isolated from a normal animal which possesses it.

5.1.2.   ISOLATION AND ESTABLISHMENT
OF STEM CELLS

15   Any mammalian stem cells which can be isolated
and maintained in vitro can potentially be used in
accordance with the present invention. Such stem cells
include but are not limited to hematopoietic stem cells
(HSC), stem cells of epithelial tissues such as the skin
and the lining of the gut, and embryonic heart muscle

20 cells.

5.1.2.1.   HEMATOPOIETIC STEM CELLS

           Any technique which provides for the isolation,
propagation, and maintenance in vitro of hematopoietic stem

25 cells (HSC) can be used in this embodiment of the
invention. Techniques by which this may be accomplished
include (a) the isolation and establishment of HSC cultures
from bone marrow cells isolated from the future host, or
(b) the isolation and establishment of HSC cultures from

30 bone marrow cells of a donor, or (c) the use of previously
established long-term HSC cultures, which may be allogeneic
or xenogeneic. Both methods (b) and (c) would be used in
conjunction with a method of suppressing transplantation
immune reactions of the future host/patient. (Such a

35 method is described in section 5.2.1, infra). The use of

previously established HSC cell cultures already expressing
a therapeutically important metabolase gene could greatly
facilitate the ease and availability of treatment in
accordance with the present invention.

5   In a particular embodiment of the present
invention, human bone marrow cells can be obtained from the
posterior iliac crest by needle aspiration. A procedure
which may be used is detailed _infra_, and is intended for
descriptive purposes only, in no way limiting the scope of
10   the present invention (see also Kodo, H., et al., 1984, J.
Clin. Invest. 73:1377-1384): Using sterile precautions,
the region of a posterior iliac crest is prepped with soap
and water and Betadine. Then the region is locally
anesthetized with 1% Xylocaine infiltration. The posterior
15   iliac crest is penetrated with an obturated needle. Ten
milliliters of marrow is aspirated into 1000 ml Collins
solution containing 10^4 units of heparin. The needle is
withdrawn. Bleeding is controlled with pressure and a
steri strip. The marrow cells are processed and cultured
20   according to Kodo et al. (1984, J. Clin. Invest. 73:1377-
1384).

           In a preferred embodiment of the present
invention, the HSCs can be isolated in highly enriched or
substantially pure form. This enrichment can be
25   accomplished before, during, or after long-term culturing,
and can be done by any techniques known in the art. Such
techniques include but are not limited to cell sorting and
antibody depletion mediated by complement. For example,
size fractionation by flow cytometry using a fluorescent
30   activated cell sorter (FACS) can be used to enrich for HSCs
among the bone marrow cell population since HSCs are
present in the large, proliferating cell pool. In
addition, a fluorescent-labeled (_e.g._ conjugated to
fluorescein) monoclonal antibody directed against a T
35   cell-surface antigen that is found in relatively low levels

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on HSCs, can be incubated with the total cell population, followed by cell sorting to separate non-labeled from labeled cells. Negative selection is also possible, by eliminating non-HSC cells. This may be accomplished by

5   binding of mAbs directed relatively specifically to non-HSC cells, followed by incubation with complement, in order to carry out complement-mediated lysis of non-HSC cells (Binz, H. and Wigzell, H., 1975, J. Exp. Med. 142:197-211). For example, human T cell-specific monoclonal antibodies (mAbs)

10 that could be used in an antibody depletion protocol include but are not limited to mAbs directed against the CD4, CD8, or CD11 T cell-surface antigens. If a cell-surface antigen that is relatively specific to HSCs is known, a fluorescent labeled mAb to such antigen could be

15 used in conjunction with FACS to specifically select for HSCs by sorting and collecting cells labeled by such antibody. For example, murine HSCs can be selected by binding of a fluorescein isothiocyanate (FITC)-conjugated anti-Thy-1 monoclonal antibody.

20   Long-term cultures of bone marrow cells can be established and maintained by using, for example, modified Dexter cell culture techniques (Dexter, T.M., et al., 1977, J. Cell. Physiol. 91:335) (modified by growth in plastic flasks rather than glass flasks) such as described in

25 Section 7.2.1, infra, or Witlock-Witte culture techniques (Witlock, C.A. and Witte, O.N., 1982, Proc. Natl. Acad. Sci. U.S.A. 79:3608-3612). Stromal cells are bone marrow fat cells, required as nurse cells, that have been shown to be necessary for the establishment of bone marrow cell

30 cultures. No T cell differentiation has been observed in such bone marrow cultures; thus long-term cultures are somewhat enriched for HSCs since mature T cells die out and no new T cells are generated. Although the murine HSC is known to be present in stromal cell layers of long-term

35 bone marrow cell cultures, we have found that they also

exist in significant number within the non-adherent
populations. Human HSCs may also be present in the non-
adherent population. If this is verified experimentally,
the non-adherent cells from modified Dexter cultures can be
5   used in accordance with the present invention for the
expression of a foreign gene. The use of the non-adherent
cell population as opposed to the stromal layer cells,
permits the expansion of the HSCs without destruction of
the cultures.

10   If the HSCs are provided by a donor, a method
for suppression of graft versus host reactivity, as well as
host versus graft reactivity, should be envisioned for use
(see Sections 5.2.1.1 and 5.2.1.2, infra.

15   5.1.2.2. STEM CELLS OF EPITHELIAL TISSUES
            Epithelial stem cells (ESCs) or keratinocytes
can be obtained from tissues such as the skin and the
lining of the gut by known procedures (Rheinwald, J.G.,
1980, Meth. Cell Bio. 21A:229). In stratified epithelial
20 tissue such as the skin, renewal occurs by mitosis of stem
cells within the germinal layer, the layer closest to the
basal lamina. Stem cells within the lining of the gut
provide for a rapid renewal rate of this tissue. ESCs or
keratinocytes obtained from the skin or lining of the gut
25 of a patient or donor can be grown in tissue culture
(Rheinwald, J.G., 1980, Meth. Cell Bio. 21A:229; Pittelkow,
M.R. and Scott, R.E., 1986, Mayo Clinic Proc. 61:771). If
the ESCs are provided by a donor, a method for suppression
of host versus graft reactivity (see Section 5.2.1.2,
30 infra) can be envisioned for use.

            5.1.3. INTRODUCTION OF THE HETEROLOGOUS
                   GENE INTO STEM CELLS
            Numerous techniques are known in the art for the
introduction of foreign genes into cells and may be used in
35 accordance with the present invention, provided that the

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necessary developmental and physiological functions of the
recipient stem cells are not disrupted.   The technique
should provide for the stable transfer of the heterologous
gene sequence to the stem cell, so that the heterologous
gene sequence is heritable and expressible by stem cell
progeny.   Techniques which may be used include but are not
limited to those listed in Table II, <u>infra</u>, which is
derived from Cline, M.J., 1985, Pharmac. Ther. 29:69-92,
incorporated by reference herein.

TABLE II

TECHNIQUES FOR METABOLASE
GENE TRANSFER TO STEM CELLS

Technique	Efficiency	References
CHROMOSOME TRANSFER:		
1. Cell fusion	10^{-6}	Ruddle and Creagan, 1975, A. Rev. Genet. 9:407
2. Chromosome-mediated gene transfer	10^{-6}	McBride and Ozer, 1973, P.N.A.S. USA 70:1258; McBride et al., 1978, P.N.A.S. USA 74:914
3. Microcell-mediated gene transfer	10^{-6}	Ege and Ringertz, 1974, Expl. Cell. Res. 7:378; Fournier and Ruddle, 1977, P.N.A.S. USA 74:319
PHYSICAL:		
1. Transfection	10^{-3} to 10^{-7}	Bachetti and Graham, 1977, P.N.A.S. U.S.A. 74:1590; Gorman et al., 1983, Science 221:551; Pellicer et al., 1978, Cell 14:133
2. Spheroplast fusion	$1-3 \times 10^{-3}$	De-Saint Vincent et al., 1981, Cell 27:267; Schaffner, 1980, P.N.A.S. U.S.A. 72:2163
3. Microinjection	0.1-1%	Capecchi, 1980, Cell 22:479

| 4. Electroporation | $*10^{-3}$ to 10^{-5} | Potter et al., 1984, P.N.A.S. U.S.A. 81:7161 |
| 5. Liposome carrier | $*2 \times 10^{-4}$ | Schaefer-Ridder et al., 1982, Science 215:166 |

VIRAL VECTORS:

| 1. Recombinant DNA viruses | 10-100% | Hamer and Leder, 1979, Cell 17:737; Hamer and Leder, 1979, Nature 281:35; Mulligan et al., 1979, Nature 277:108 |
| 2. Recombinant RNA viruses | 10-100% | Mann et al., 1983, Cell 33:153; Shimotohno and Temin, 1981, Cell 26:67; Tabin et al., 1982, Mol. Cell. Biol. 2:426 |

* Rough estimation of lower limit of range

5.1.3.1. PHYSICAL METHODS OF TRANSFER

Transfection, or DNA-mediated gene transfer, can be used to introduce the metabolase gene into the mammalian stem cell in accordance with the present invention. The transfection can be carried out by any techniques known in the art including, but not limited to, the use of calcium-phosphate precipitation (Wigler, M., et al., 1978, Cell 14:725-731), DEAE-dextran (Hermonat, P.L. and Muzyczka, N., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6466-6470), or treatment with polybrene and dimethyl sulfoxide (Kawai, S. and Nishizawa, M., 1984, Mol. Cell. Biol. 4:1172).

Electroporation is a preferred method of introducing the metabolase gene (see Section 8.2, infra). Electroporation involves the exposure of target cells to high field strength electrical pulses which reversibly permeabilize cell membranes (Neumann, E. and Rosenheck, K., 1972, J. Membr. Biol. 10:279-290; Serpersu, E.H., et al.,

1985, Biochim. Biophys. Acta 812:779-785). This procedure
has major advantages over other transfer methods, including
high frequency of introduction, relative ease of the
method, and the lack of introduction of viral genes (in
5   viral transfer methods) into the target cell genome. In a
particular embodiment, since it is a more efficient
technique than tranfection for gene transfer into
nonadherent cells which grow in suspension culture, it can
be useful for the introduction of the metabolase gene into
10   a nonadherent HSC population grown in..modified Dexter cell
culture, such as described in Section 5.1.2.1, supra.

                5.1.3.2. VIRAL-MEDIATED TRANSFER
                Any viral vector capable of stably transferring
15   the metabolase gene to the mammalian stem cell without
significant undesirable side effects can be used in
accordance with this embodiment of the invention. Such
vectors include but are not limited to those derived from
parvoviruses such as adeno-associated viruses (such as
20   described in Sections 6, 7, infra), papovaviruses such as
bovine papilloma viruses, herpes simplex viruses,
retroviruses, etc. (See e.g., Tratschin, J.-D., et al.,
1985, Mol. Cell. Biol. 5:3251; Campo, M.S., 1985, in DNA
Cloning: A Practical Approach, Vol. II., IRL Press, Oxford
25   UK, p. 213; Hock, R.A. and Miller, D., 1986, Nature
320:275-277; Cline, M.J., 1985, Pharmac. Ther. 29:69-92.)
Papilloma viruses are useful as vectors because of their
unique ability to persist exclusively as multicopy,
nonintegrated plasmids in infected mammalian cells.
30   Retroviruses possess several properties which are desirable
for gene transfer vectors: (a) normal replication of
retroviruses involves a stable insertion of a DNA copy of
the viral genome into the host genome, (b) retroviruses
have a broad host range and theoretically can infect most
35   if not all cell types, (c) retrovirus infection is usually

not toxic to the cell, and (d) large sequences of
heterologous DNA can be packaged within the retroviral
virion. Despite these properties, retroviruses have
disadvantages for use in gene therapy, because they are
5   potentially pathogenic and possess strong enhancer/promoter
regions in their long terminal repeats which affect normal
regulatory elements which provide for gene expression.
Like retrovirus, adeno-associated virus (AAV) has several
features which are desirable for gene transfer vectors:
10   (a) AAV has a broad host range and can infect numerous cell
types, (b) AAV normally produces an efficient latent
infection with significant proviral integration, (c) AAV is
non-pathogenic and is not known to cause secondary
complications, and (d) replication, packaging and
15   integration of AAV requires only that terminal repeats
remain intact. However, AAV can be constructed to contain
at maximum approximately 4.5 kilobase pair of foreign DNA.
        A virus vector can be constructed which contains
a metabolase gene capable of expressing a functional enzyme
20   within the stem cell progeny. Such a vector can be
constructed by recombinant DNA techniques such as described
in Section 5.1.1, supra. Preferably, the vector is
defective in that it lacks viral functions necessary for
production of infectious progeny, even in the presence of
25   endogenous cellular viral sequences. However it must
retain functions necessary for stable gene transfer to the
desired stem cell, such as cellular adsorption, penetration
and integration into the genome of the specific host stem
cell (or stable existence as an autonomously replicating
30   DNA molecule). Preferably, before proceeding with viral-
mediated transfer, long-term studies are undertaken to
demonstrate the absence of any detrimental side effects.
For example, in using retroviral vectors, it is necessary
to unambiguously determine that no infectious virus
35   particles are generated and that no endogenous cellular

oncogenes are insertionally activated. It is also imperative to minimize the possibility of insertional inactivation of a necessary cellular gene upon integration of the virus vector carrying the metabolase gene. Thus vectors which do not insert at multiple chromosomal sites are preferred. Care should be taken to exclude pathogenic helper viruses or other nondefective viruses which can cause disease or disorders.

5.1.3.3. CHROMOSOME TRANSFER TECHNIQUES

Chromosome transfer techniques are advantageous in that the transferred gene is in its native form with its natural regulatory elements and structure. Such a transfer technique may have advantages for targeting to specific chromosomal sites that have been shown to promote substantial expression (Kucherlapati, R.S., et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:3153-3157). This technique would be useful, for instance, in transferring a metabolase gene from one established HSC line to another that has, for example, different transplantation antigens.

5.1.4. SELECTION OF RECOMBINANT STEM CELLS EXPRESSING THE HETEROLOGOUS METABOLASE GENE

Stem cells successfully incorporating the metabolase gene and capable of expressing its encoded enzyme will be selected prior to use in reconstitution experiments. Selection of the recombinant stem cell expressing the heterologous metabolase gene can depend on the properties of the gene itself, or on the physical, immunological, or functional properties of its encoded product. In a preferred embodiment, the stem cell population can be screened for expression of metabolase activity. In this embodiment, stem cells should be incubated with a substrate of the enzyme being selected for (i.e., the metabolite or an analogue thereof) in conjunction with a method of identifying or isolating those cells capable of substrate

alteration or degradation. Where metabolase (e.g., oxalase)
activity results in a breakdown of substrate to CO_2, in a
preferred embodiment, the metabolase activity and resulting
CO_2 release can be detected by incubation with a substrate
5 containing radiolabelled carbon, followed by acidification
to cause atmospheric release of radioactive CO_2 and exposure
of vicinal photographic film (see Section 8.3, infra). In
another embodiment of the invention involving selection for
oxalase activity, stem cells can be selected in calcium-free
10 media containing a lethal concentration of potassium-
oxalate. (Calcium oxalate should not be used since it
precipitates out of solution.) Thus, only those cells which
express a functional oxalase gene will survive. In a
specific embodiment involving selection for uricase
15 activity, media can contain a lethal concentration of uric
acid or an analogue, in which only stem cells expressing a
functional uricase will survive. If a substrate of the
metabolase is chromogenic, the assay may be on the basis of
color development. If an antibody to the enzyme is
20 available, it may be incubated with the cells to select for
bound antibody by labeling it or a second anti-antibody
molecule. To confirm for the presence of the gene itself,
DNA can be isolated from a subpopulation of the stem cells
and screened by hybridization to a labeled cloned metabolase
25 gene (see Southern, E., 1980, Meth. Enzymol. 69:152). Many
different assays are possible and within the scope of the
present invention.

In a particular embodiment employing HSCs,
recombinant HSCs can be selected in the presence of media
30 containing the metabolite substrate. Once selected, the
HSCs can be placed on primary stromal cells for expansion.
The possibility exists that the effects of selective media
are highly reversible following short-term incubation in the
selective media. For this reason, a second approach to the
35 selection of transfected HSCs can be used. This approach

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uses stromal cells which have also been transfected with the
metabolase gene. Such stromal cell cultures can be prepared
in advance of the transfection of any HSC population.
Transfected stromal cells will be selected for in the same
5 way as HSCs, for example, by use of medium supplemented with
a metabolite concentration lethal for normal, non-
transfected cells. Thus, after selection of HSCs, the HSCs
can be propagated on stromal cells under conditions of
continual selection. If the isolated and selected HSC
10 population is not obtained in large enough quantities to
allow further propagation after replacement on stromal
cells, total bone marrow can be transfected and selected
before enrichment for HSCs.

15   5.2.   INTRODUCTION AND ESTABLISHMENT OF
                RECOMBINANT STEM CELLS IN THE HOST
         Different protocols are envisioned for use
depending on the source of the recombinant stem cell.
Preferably, the recombinant stem cell for use in the present
invention is derived from cultures initially obtained from
20 the cells of the future host/patient itself/himself. If
this is not feasible, the stem cells obtained from a
histocompatible donor may be used. Alternatively, it may
facilitate treatment to maintain established stem cell
cultures that have already incorporated the desired
25 metabolase gene. Thus, these recombinant stem cells would
not be histocompatible for all hosts. In using stem cells
which are not derived from the future host, a protocol
should be employed to avoid immune responses to
transplantation antigens. For example, a protocol which
30 mediates suppression of graft versus host reactivity, such
as described in Section 5.2.1, infra, can be used. In a
particular embodiment using hematopoietic stem cells,
procedures can also be employed which use donor stem cell
populations from long-term bone marrow cultures which do not
35 allow T cell differentiation, such as modified Dexter or

Witlock Witte cultures. These cultures are substantially
devoid of mature T lymphocytes, since the mature T cells die
out while no new T cells are generated. The preferred use
of nonadherent cells from such cultures allows the isolation

5   of the stem cells for introduction into the host without
destruction of the cultures.

The recombinant cell population can be
substantially enriched, or further enriched, for the desired
stem cell population before introduction into the host, by

10 procedures such as fluorescent-activated cell sorting on the
basis of cell size or antibody-binding, or antibody
depletion mediated by complement (see Section 5.1.2.1,
supra).

Recombinant stem cells may be introduced by a

15 variety of methods. The stem cells may be introduced by
injection by various routes, including but not limited to
intradermal, intramuscular, intravenous, intraperitoneal,
intranasal, etc. In a particular embodiment employing human
hematopoietic stem cells, the recombinant HSCs are

20 preferably introduced into the host by intravenous injection
into any peripheral vein. In determining the amount of
recombinant stem cells to be introduced into the host, it is
preferable to take into account the catalytic efficiency of
the recombinant cells (which can be determined, for example,

25 by in vitro assay), and the recipient's metabolic need. As
an example, in the case of congenital hyperoxaluria, a
production of 200 mg of oxalate per day would require a mass
of cells with sufficient activity to catalyze three times
that amount to deal with any loss of activity due to

30 feedback inhibition. Simple kinetic considerations indicate
that within several orders of magnitude, the therapeutic
cell number would be 10^{11}, although it may range down to
10^3. The determination of the optimal amount of recombinant
stem cells for introduction into the host will also depend

35 on whether the metabolase is secreted or not. For instance,

recombinant stem cells which secrete the metabolase would be
required in lesser amount than recombinant stem cells from
which the metabolase must diffuse out in order to exert its
effect. In some studies, between 1.5-6.0 x 10^8 freshly
5   explanted bone marrow cells per kilogram have been
introduced into lethally-irradiated or aplastic anemic human
hosts (Thomas et al., 1977, Blood 49:511; Storb et al.,
1977, N. Eng. J. Med. 296:61). In a preferred embodiment,
approximately 10^6 to 10^{11} cultured bone marrow cells, or
10   approximately 10^3 to 10^{11} purified HSCs, which contain the
metabolase gene can be introduced into a human host. In
another embodiment employing epithelial stem cells,
recombinant skin cells may be applied as a skin graft onto
the patient. Such an embodiment may be preferred for use
15   where it is important to avoid a systemic immune response,
since the metabolase gene is expressed at a localized site.
Recombinant epithelial cells may also be introduced by
injection by various routes, with intradermal (subcutaneous)
injections preferred. The amount of epithelial stem cells
20   envisioned for use depends on the enzyme system and the
concentration of the enzyme needed in the host. Although
approximately less than 10^9 epithelial stem cells are
envisioned for use in injection at any one injection site,
the optimal amounts can be determined by experiments in
25   vitro, and in vivo with various animal models.

5.2.1.   SUPRESSION OF DELETERIOUS IMMUNE RESPONSES

The use of autologous recombinant stem cells is
preferred, since it would avoid problematic effects such as
30   lack of engraftment, graft vs. host disease, and host vs.
graft disease. However, the use of previously established
recombinant stem cell cultures containing the desired
metabolase gene, which may be allogeneic, can greatly
facilitate treatment. When using a non-autologous system,
35   in order for the recombinant stem cells of the present

invention to be stably and safely established within the
host, it is necessary to suppress deleterious immune
responses such as those which may be mediated by the host
immune system against the recombinant progeny cells (host
5 vs. graft reactivity) and possibly their encoded
heterologous metabolases, and those immune responses which
may be mediated by recombinant HSC progeny against the host
(graft vs. host reactivity).

10   5.2.1.1.   SUPPRESSION OF GRAFT VS. HOST REACTIVITY

         In an embodiment of the invention employing non-
autologous recombinant HSCs, the use of a suppressor-inducer
cell which facilitates successful engraftment and helps
prevent the development of graft vs. host (GVH) disease is
15 preferred. Culturing the recombinant HSCs with a suppressor
cell (e.g., 24 hours prior to introduction into the host)
can enhance the stability of recombinant HSC establishment
and reduce the risk of GVH disease. Such a cell has been
described in human bone marrow (Mortari, F., et al., 1986,
20 J. Immunol. 137(4):1133-1137; McGarry, R.C. and Singhal,
S.K., 1982, Immunol. 46(2):387-394). Its presence in other
species is well-established (see e.g., Skowron-Cendrzak, A.
and Ptak, W., 1976, Eur. J. Immunol. 6:451; Murgita, R.A.,
et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:2897, Peeler,
25 K., et al., 1983, J. Immunol. 17:443; Jadus, M. and Peck,
A.B., 1984, Scand. J. Immunol. 20:81, Strober, S., et al.,
1987, J. Immunol. 138:699), where it has been identified as
a monocyte which elicits its suppressor activity in part
through the secretion of soluble mediators capable of
30 activating the T suppressor limb of the immune response
(Basset, M., et al., 1977, J. Immunol. 119:1855; Argyris,
B.F., 1981, Cell. Immunol. 62:412; Peeler, K.L., et al.,
1983, Fed. Proc. 42:935; Jadus, M.R. and Peck, A.B., 1986,

35

Scand. J. Immunol. 23:35). The experiments detailed in Section 8, *infra*, describe the use of such a suppressor cell in the prevention of GVH in a murine model.

5   5.2.1.2.   SUPPRESSION OF HOST VS. GRAFT REACTIVITY

Where the recombinant stem cells for use in the present invention are non-autologous, a method of suppressing host vs. graft reactivity should also be envisioned for use. In a preferred embodiment, a moderate

10 immunosuppression of the host can be induced. Immunosuppression can be induced by any methods known in the art including but not limited to irradiation, drug administration (*e.g.* prednisone, azathioprine, cyclosporin, cytoxan), antibody administration (*e.g.* OKT3 monoclonal

15 antibody), etc. For example, a protocol using radiation can consist of approximately 100 rads of total lymphoid irradiation each day for 14 days.

       5.2.2.   DEMONSTRATION OF EXPRESSION OF
             THE HETEROLOGOUS GENE IN VIVO

20   Successful establishment within the host can be demonstrated by showing the presence and detectable expression of the heterologous gene among the known progeny cells of the stem cell. Ideally, the metabolase activity is detectable among the various cell lineages known to derive

25 from the stem cell. In a particular embodiment involving the use of HSCs, blood-borne cells of the host such as reticulocytes, granulocytes, and cells residing in lymphoid organs and tissues, should express the metabolase gene.

       Incorporation of the metabolase gene into the

30 genome of the stem cell progeny can be shown by nucleic acid hybridization to genomic DNA isolated from the cell progeny (*e.g.*, Southern, E., 1980, Meth. Enzymol. 69:152). For example, DNA of stem cell progeny can be isolated from blood cells or cells residing in lymphoid tissue (for HSC

35 progeny), or from a skin sample (for ESC progeny), or other

-39-

suitable tissue samples by standard techniques (Maniatis et
al., 1982, Molecular Cloning: A Laboratory Manual, Cold
Spring Harbor Laboratory, New York). For example, the
following procedure may be used to obtain DNA of HSC

5   progeny, and is intended for descriptive purposes only, in
no way limiting the scope of the invention: Blood cells can
be collected by centrifugation, placed in liquid nitrogen,
and ground to a fine powder. The resulting cell debris can
be suspended in buffer containing EDTA, sodium

10   dodecylsulfate, and a protease such as proteinase K and
incubated at 65°C for 16 hours to allow digestion. After
protease digestion, the DNA can be extracted, precipitated,
collected and redissolved before restriction endonuclease
digestion, electrophoresis, and "Southern" transfer to a

15   filter such as nitrocellulose or Zetabind nylon filters
(Southern, E., 1980, Meth. Enzymol. 69:152). DNA on the
filter can then be tested for the presence of the metabolase
gene by hybridization to a labeled metabolase gene or
fragment thereof. The label can be a radioisotope, in which

20   case the binding to the metabolase gene can be assayed by
exposure to X-ray film. Other labels and assays can be
used, including but not limited to enzymatic labels with
chromogenic substrates.

     Functional expression of the metabolase gene in

25   stem cell progeny can also be assayed for directly.
Isolated progeny cells grown in vitro can be tested for
expression of the metabolase gene by assays based on its
functional, physical, or immunological properties. For
example, if an antibody to the enzyme is available, a

30   labeled antibody molecule could be used to screen among
progeny cell protein for bound antibody. (Alternatively, an
anti-enzyme antibody plus labeled anti-antibody molecule
could be used.) In a preferred embodiment, the assay can
test for alteration or degradation of the substrate

35   metabolite. For example, where such degradation leads to

production of CO_2, an assay such as that described in
Section 8.3, _infra_, can be used to detect release of CO_2
from radiolabeled substrate by acidification of the cell
culture and exposure to photographic film. In an
5 alternative embodiment, progeny stem cells can be cultivated
in media containing a lethal concentration of the metabolite
or analogue thereof. Only those cells which express the
heterologous gene and are thus capable of metabolizing the
substrate will survive. In a particular embodiment assaying
10 for oxalase activity of HSC progeny, blood cells can be
grown in medium supplemented with a lethal level of
potassium-oxalate. In another particular embodiment
assaying for uricase activity, the medium can be
supplemented with a lethal amount of uric acid or a salt
15 thereof. If the substrate is chromogenic, an assay for
color development can be used. A quantitative determination
of metabolase activity can also be made. For example, in a
specific embodiment assaying for oxalase activity, stem cell
progeny can be incubated with ^{14}C-oxalate. The metabolism
of oxalate can then be estimated from measurements of ^{14}C-
20 CO_2 produced by oxalate degradation (Allison, M.J., and
Cook, H.M., 1981, Science 212:675). Quantitative
determinations of oxalate degradation products can also be
made by manometric determinations of CO_2 (Bergmeyer, U.H.,
ed., 1974, Methods of Enzymatic Analysis, 2nd Ed., Vol. 3,
25 Academic Press, New York, pp. 1542-1546), or photometric
determinations of formate production (_id._), potentiometric
determinations of CO_2 with a CO_2 electrode (Yao, et al.,
1975, Bioelectrochemistry and Bioenergetics 2:348-350),
double enzyme protocols based on formic acid production
30 (Hodgkinson, A., 1977, Oxalic Acid in Biology and Medicine,
Academic Press, New York, p. 96), etc. In a particular
embodiment assaying for uricase, a quantitative
determination of uricase activity can be made by
spectrophotometric measurements of the time rate of urate
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uptake from an _in_ _vitro_ physiological solution containing
recombinant cells, or by examining the time rate of urate
disappearance from media harvested from recombinant cells,
among other methods. This can be accomplished by standard

5 techniques known in the art. As one example, kits for the
measurement of uric acid are commercially available (Sigma
Chemical Company, St. Louis, MO., Cat. No. 685), and can be
used. Many different assays are possible and within the
scope of the invention.

10   Expression of the desired metabolase activity can
also be demonstrated by the successful metabolism _in_ _vivo_ of
the substrate metabolite. Human patients or animals that
are undergoing treatment in accordance with the present
invention should be monitored frequently for determinations

15 of the level of the metabolite at the appropriate _in_ _vivo_
site, such as the blood, urine, tissue extracts, etc. A
decrease in metabolite levels and/or the resultant diseases
or disorders is indicative of successful expression of the
metabolase gene.

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5.3. TREATMENT OF DISORDERS DUE TO ACCUMULATIONS OF OR INCREASES IN METABOLITES

       Any disorder in mammals resulting from an
accumulation of or increase in a metabolite that is a
substrate for a heterologous enzyme whose activity results

25 in nontoxic products in the heterologous host, can be
subjected to the methods of treatment or prevention as
provided for by the present invention. Metabolites which
are substrates for heterologous enzymes which can be used in
accordance with the present invention include but are not

30 limited to oxalic acid, uric acid, a phenylketone,
phenylpyruvic acid, phenylethylamine, a porphyrin or related
molecule, delta-amino levulinic acid, testosterone, and
cholesterol. The detrimental increase in the metabolite can
take many forms, including but not limited to localized and

35 systemic concentration increases and precipitate formation.

In a particular embodiment, a metabolase which selectively alters or degrades uroporphyrin I or coproporphyrin can be used to treat a porphyria (e.g. congenital erythropoietic, intermittent acute, hereditary coproporphyria, variegative,

5   and cutanea tarda) (Meyer, U.S. and Schmid, R., 1978, The porphyrias, in The Metabolic Basis of Inherited Disease, Stanbury, J.B., et al., eds., 4th Ed., Ch. 50, McGraw-Hill Book Co., New York, pp. 1165-1220). In another embodiment, a metabolase which selectively alters or degrades

10 phenylpyruvic acid or phenylethylamine can be used to treat phenylketonuria (Tourian, A.Y., and Sidbury, J.B., 1978, Phenylketonuria, in The Metabolic Basis of Inherited Disease, supra, pp. 240-255). In another embodiment, a metabolase that selectively degrades or inactivates

15 testosterone can be used to lower the amount of this hormone, for the palliation of prostate carcinoma (Catalona, W.J., 1986, Carcinoma of the prostate -- clinical stage C, in Current Urologic Therapy, Kaufman, J.J., ed., W.B. Saunders Co., Philadelphia, pp. 300-301). This would be a valuable alternative to other methods of reducing

20 testosterone levels such as castration or female hormone administration. In yet another embodiment, a metabolase which degrades cholesterol or alters cholesterol (e.g. lecithin:cholesterol acyltransferase) can be used to treat cholesterol-related disorders including but not limited to

25 hypercholesterolemia, atherosclerosis, and familial lecithin:cholesterol acyltransferase deficiency (Gjone, E., et al., 1978, Familial lecithin cholesterol acyltransferase deficiency, in The Metabolic Basis of Inherited Disease,

30 supra, pp. 589-603).

5.3.1. OXALATE DISORDERS

       Disorders resulting from increases in oxalate which can be treated according to the present invention

35 include but are not limited to those listed in Table III,

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<u>infra</u>, which is derived from Hodgkinson, A., 1977, Oxalic
Acid in Biology and Medicine, Academic Press, New York, pp.
217-241, which is incorporated by reference herein.

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TABLE III

DISORDERS WHICH CAN BE TREATED BY
OXALASE GENE THERAPY

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OXALOSIS
    Acute oxalic acid toxicity
      Local corrosive effects

10
      Systemic effects
      Renal insufficiency
      Tetany

    Renal failure

15

    Calcium oxalate crystal formation

OXALATE UROLITHIASIS
    Kidney stones

20

HYPEROXALURIA
    Primary hyperoxaluria Type I
    Primary hyperoxaluria Type II

25   OXALATE DISORDERS INDUCED BY:
    Ethylene glycol
    Diethylene glycol
    Xylitol
    Methoxyflurane

30
    Aspergillosis
    Ascorbic acid
    Intestinal disease

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-45-

There are many possible embodiments of this aspect of the invention. In one particular embodiment for treating or preventing the systemic effects of oxalosis, blood cells derived from recombinant HSCs expressing a bacterial oxalase gene can metabolize the oxalate in the blood, reducing or preventing such symptoms in humans as weak pulse, low blood pressure and body temperature, muscular and abdominal pain, and convulsions. In addition, decreasing an abnormally high systemic concentration of oxalate in this fashion may prevent calcium oxalate crystal formation and renal failure. Recombinant epithelial stem cells, such as those which form skin cells, can be used in skin grafts to provide for the diffusion of oxalase activity through cell layers into the circulation, or for a more localized treatment. In another embodiment, cells derived from recombinant stem cells of the lining of the gut or from recombinant HSCs, may be especially useful in treating or preventing oxalosis and concomitant effects such as renal failure and crystal formation caused by the metabolic production of oxalate from ingested compounds such as ethylene glycol, diethylene glycol, xylitol, or ascorbic acid, or from the anaesthetic methoxyflurane. Infection by the oxalic-acid-producing fungi of Aspergillus spp. may cause deposits of calcium oxalate at the site of infection (the lungs) and renal oxalosis and impairment, which can also be treated by the present invention. Genetic defects such as primary hyperoxaluria, Types I and II, are especially suited to oxalase gene therapy, since the disorders are chronic ones that can be stably corrected by the procedures of the present invention. Hyperoxaluria in patients with intestinal disease has been shown to occur from increased absorption of dietary oxalate (Chadwick et al., 1973, N. Engl. J. Med. 289:172-196). Cells expressing oxalase which are derived from epithelial or hematopoietic stem cells may be especially useful in correcting this defect.

Where the predisposing or causative factors of disorders due to increased oxalate are known and present, oxalate gene therapy, as provided for by the present invention, may also be employed as a prophylactic measure.

5 For example, patients undergoing treatment with steroids, immunosuppressive drugs, or cytotoxic agents are more prone to developing Aspergillosis, and it may be desirable to circumvent this detrimental side-effect. Intestinal disease has been reported to be the most common cause of

10 hyperoxaluria in man (Smith, L.H., and Hofmann, A.F., 1974, Gastroenterology 6: 1257-1261). Thus, oxalate gene therapy may be desirable for patients who have, for example, inflammatory ileal disease or resection of the terminal ileum, to treat hyperoxaluria and prevent associated

15 developments such as the formation of calcium oxalate renal calculi.

In another specific embodiment of the present invention, the recombinant stem cell progeny expressing a heterologous oxalase gene in accordance with the present

20 invention can be used in the treatment or prevention of livestock poisoning due to oxalate ingestion. This would allow plants with high-oxalate content such as Halogeton and Setaria to be used as foodstuffs without induction of oxalate toxicity. A recombinant epithelial stem cell,

25 derived from the lining of the gut, may be preferred for use.

5.3.2. URIC ACID DISORDERS

Disorders resulting from increases in uric acid

30 which can be treated according to the present invention include but are not limited to those listed in Table IV, infra, which is derived from Gutman, A.B. and Yu, T.-F., 1968, Am. J. Med. 45:756-779, which is incorporated by reference herein.

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TABLE IV

DISORDERS WHICH CAN BE TREATED BY
URICASE GENE THERAPY

Gout

Renal failure

Uric acid nephrolithiasis:

 kidney stones, sand, gravel

For example, in particular embodiments, a
metabolase which selectively degrades uric acid or alters
uric acid to an innocuous form such as allantoin, can be
used to treat gout or uric acid stones (Wyngaarden, J.B. and
Kelley, W.N., 1978, Gout, in The Metabolic Basis of
Inherited Disease, Stanbury, J.B., et al., eds., McGraw-Hill
Book Co., New York, pp. 916-1010).

Where the predisposing or causative factors of the
uric acid disorders are known and present, uricase gene
therapy, in accordance with the present invention, can also
be used as a prophylactic measure. For example, if some of
the conditions listed in Table V, infra, are present,
uricase gene therapy may be used to avert the formation of
uric acid calculi.

TABLE V

ETIOLOGY OF URIC ACID NEPHROLITHIASIS[1]

I. Idiopathic
> 1. Sporadic
> 2. Genetically transmitted

II. Associated with hyperuricemia
> 1. Inborn errors of metabolism
>>   Primary gout
>>   Lesch-Nyhan syndrome
>>   Glycogen storage diseases
> 2. Myeloproliferative and other neoplastic diseases
> 3. Undetermined causes

III. Associated with dehydration due to excessive extrarenal water loss
> 1. By way of the skin
> 2. By way of the gastrointestinal tract

IV. Associated with hyperuricosuria without significant hyperuricemia.
> 1. Uricosuric drugs
> 2. Ingestion of excess purines and protein
> 3. Inherent or acquired defect in tubular reabsorption of uric acid.

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[1] Gutman, A.B. and Yu, T.-F., 1968, Am. J. Med. 45:756-779.

In patients prone to kidney stone formation, gene therapies in accordance with the present invention for both oxalate and uric acid disorders may be used, since oxalates are a common contaminant of uric acid stones (Gutman and Yu,

5 _supra_, p. 762).

5.3.3.   SYNTHETIC METABOLITE CHELATORS

In another embodiment of the invention, molecular modelling of the binding site of a metabolase enzyme can be

10 done in order to determine the desired molecular structure for production of synthetic metabolite chelators. In a particular aspect of this embodiment, the DNA sequence of the cloned metabolase gene can be used for computer modelling studies based on the derived amino acid sequence.

15 Computer modelling (Fletterick, R. and Zoller, M. (eds.), 1986, Computer Graphics and Molecular Modelling, _in_ Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, New York) can provide theoretical three-dimensional images of the metabolase molecule. Information

20 derived from computer modelling studies can thus be used to predict the higher-order structure of the enzyme at its binding site. Molecules can be synthesized which are predicted to have a similar conformation, and which thus will putatively bind the metabolite. Synthetic metabolite

25 chelators obtained in this fashion can be used to treat or prevent a disorder due to an increased concentration or accumulation of a metabolite, by introduction into or onto the host, _e.g._ by injection into the patient's circulation, ingestion of the chelator, etc. In a preferred aspect of

30 this particular embodiment, the synthetic chelator can also have metabolase activity.

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5.4. EXPRESSION OF IMMUNOGLOBULIN GENES
IN RECOMBINANT STEM CELL PROGENY

The procedures described supra can also be applied to an alternative embodiment of the invention in which heterologous immunoglobulin genes are transferred to a stem cell for expression in the stem cell progeny. For example, functional immunoglobulin genes derived from a hybridoma which produces a monoclonal antibody may be transferred so that the stem cell progeny produce a functional monoclonal antibody molecule. If the monoclonal antibody is a neutralizing antibody specific for an epitope of a pathogenic microorganism, the generation of stem cell progeny expressing the monoclonal antibody can be especially valuable in preventing or treating diseases or disorders caused by the microorganism. Such microorganisms include but are not limited to bacteria, viruses, and fungi. In a particular embodiment, recombinant epithelial stem cell progeny (e.g. keratinocytes) which produce a monoclonal antibody directed against Pseudomonas spp. can be used as a dressing to cover burns. This procedure can prevent or treat Pseudomonas infection which is a major complication of burns.

In this embodiment of the invention, selection of recombinant stem cells can preferably be on the basis of antibody expression. There are many techniques known in the art by which such selection may be done, including but not limited to those based on labeled antigen binding, labeled anti-antibody binding, etc. In a particular embodiment, the selection can be for soluble antibody secretion.

It may not be necessary for the entire heterologous immunoglobulin genes to be present in the recombinant stem cell progeny, as long as assembly and secretion and/or cell-surface expression of a functional binding domain occurs which results in the desired anti-bacterial, anti-viral, or anti-fungal activity.

5.5. ANTIBODIES REACTIVE WITH METABOLASES

Antibodies to metabolases can be of great value in the isolation, detection, and characterization of the metabolase proteins, and in immunoassays.

Antibodies can be produced which recognize a metabolase protein. Such antibodies can be polyclonal or monoclonal.

Various procedures known in the art may be used for the production of polyclonal antibodies. Various host animals can be immunized by injection with a metabolase protein, or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), RIBI, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

A monoclonal antibody to a metabolase protein can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256:495-497) (see, e.g., Section 10, infra), and the more recent human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72) and EBV-transformation technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')$_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be

generated by reducing the disulfide bridges of the $F(ab')_2$
fragment, and the Fab fragments which can be generated by
treating the antibody molecule with papain and a reducing
agent.

5   Antibodies reactive with a metabolase can be used
in immunoassays to detect, localize, or measure the amount
of the metabolase protein in a sample. The immunoassays
which can be used include but are not limited to competitive
and non-competitive assay systems using techniques such as
10 radioimmunoassays, ELISA (enzyme linked immunosorbent
assay), "sandwich" immunoassays, precipitin reactions, gel
diffusion precipitin reactions, immunodiffusion assays,
agglutination assays, complement-fixation assays,
immunoradiometric assays, fluorescent immunoassays, protein
15 A immunoassays, and immunoelectrophoresis assays, to name
but a few.

          Antibodies, preferably monoclonal, can also be
used in the isolation and/or characterization of the
metabolase which they recognize, by procedures known in the
20 act, e.g., immunoaffinity chromatography,
immunoprecipitation, etc.

6. EXAMPLE: GENE TRANSFER INTO MURINE HEMATOPOIETIC STEM
    CELLS MEDIATED BY AN ADENO-ASSOCIATED VIRUS VECTOR

          We describe here the transduction of murine
25 hematopoietic progenitor cells with the dominant selectable
neomycin drug resistance (Neo) gene using a recombinant
adeno-associated virus (AAV) vector. Successful transforma-
tion of progenitor cells to drug resistance was determined
to be approximately 1.5% by colony formation in the presence
30 of geneticin (G-418) sulfate.

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6.1. THE RECOMBINANT AAV VECTOR

Recently, Hermonat et al. (Hermonat, P.L., et al., 1984, J. Virol. 51:329-339) described the production of recombinant AAV virus stocks. As depicted in Fig. 1, a 1.8

5   kilobase pair (kb) DNA fragment containing the SV40 early promoter region and the bacterial neomycin resistance gene (Neo) was inserted into the AAV deletion mutant dl52-91, to generate the recombinant dl52-91/neo. To produce the recombinant virus stocks, 5 micrograms of dl52-91/neo

10 plasmid DNA and 0.5 micrograms of ins96/lambda-M plasmid DNA were cotransfected into adenovirus-2 (AD-2) infected KB cells or HeP-2 cells by using DEAE-dextran (Hermonat, P.L. and Muzyczka, N., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6466-6470). The ins96/lambda-M plasmid is an insertion

15 mutant which contains the cap and lip genes required for capsid production, two genes deficient in dl52-91/neo but is too large to be packaged itself (Hermonat, P.L., et al., J. Virol. 51:329-339). Furthermore, only chromosomal recombinant AAV/Neo provirus present in tandem appears to be

20 rescuable. Two days after transfection, the cells were frozen and thawed three times and passed through a 0.45-micron filter to remove cellular debris. The contaminating AD-2 helper virus was inactivated by heating the virus stock at 56°C for two hours. The resulting recombinant viral

25 stock, referred to as AAV/Neo, was shown to infect human and murine cell lines and impart resistance to the antibiotic geneticin (G-418) sulfate with an efficiency of 0.4-10% (Hermonat, P.L. and Muzyczka, N., 1984, Proc. Natl. Acad. Sci. U.S.A. 81: 6466-6470). Similar transformation efficiencies have been reported by Tratschin and co-workers

30 (Tratschin, J.D., et al., 1985, Mol. Cell. Biol. 5:3251-3260) using independently-derived recombinant AAV stocks.

35

6.2. TRANSFER OF NEOMYCIN RESISTANCE TO BONE MARROW CELLS

The purpose of this study was to determine if the recombinant AAV/Neo vector could also transduce hematopoietic progenitor cells from freshly explanted bone marrow.

5   Adult B10.BR/cd bone marrow cells were prepared by gently flushing the lumens of freshly explanted femurs and tibias. The tissue was dispersed to a single cell suspension and co-cultured for two hours with the AAV/Neo recombinant vector at a multiplicity of infection (moi) of 1 or 10 at

10   37°C. Positive transformation to neomycin resistance was determined in a colony forming unit assay based on the ability of infected cells to form colonies in soft agar containing 1.2 mg/ml G-418 sulfate. Bone marrow cells not infected with the AAV/Neo vector were unable to produce

15   colonies in the presence of G-418 sulfate; therefore, any colonies resulting from proliferation of colony forming units (CFUs) indicated successful transduction by AAV/Neo. As shown in Table VI, G-418 sulfate resistance was conferred on 0.5-1.5% of the potential CFUs contained in the bone

20   marrow following infection with the AAV/Neo vector.

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TABLE VI

TRANSFER OF NEOMYCIN RESISTANCE TO BONE MARROW CELLS USING A RECOMBINANT AAV VECTOR[1]

Expt. No.	Treatment		Moi	Plates	Colonies	Efficiency (%)
	AAV/Neo	(G-418) Sulfate				
1	−	+	.	2	0	0
	+	−	10	2	74	100
	+	+	10	2	1	1.3
2	−	+	.	2	0	0
	+	−	10	4	200	100
	+	+	10	4	3	1.5
3	−	+	.	5	0	0
	+	−	5	5	385	100
	+	+	1	5	2	0.5
	+	+	10	5	2	0.5

[1]Bone marrow cells were co-cultured with the recombinant AAV/Neo vector at a moi of 1 or 10 for 2 hours at 37°C, pelleted by centrifugation and the unbound AAV/Neo poured off with the supernate. The cells were then cultured in 1 ml soft-agar (DMEM, 10% fetal calf serum, 5% mouse L-cell conditioned media, 0.35% agar w/v) at a concentration of 0.1×10^6 cells/ml. Geneticin (G-418) sulfate was added to the appropriate cultures 24 hours after seeding to a concentration of 1.2 mg/ml. The plates were viewed for the presence of viable colonies at day 10.

The G-418 sulfate resistant colonies appeared normal and compact (Fig. 2). Colonies grew to contain several hundred cells and mainly appeared to be mixed GM or GEM colonies. The AAV/Neo recombinant virus is thus capable of transferring exogenous DNA to progenitor cells of the hematopoietic system. The small number of cells present in each colony prevented direct testing by the hybridization methods of Southern (1975, J. Mol. Biol. 98:503-517) for incorporation of the AAV/Neo into the host genome.

In the protocol used here, G-418 sulfate selection was not initiated until 24 hours after infection of the bone marrow cells with AAV/Neo. Previous data with cell lines indicated that delaying G-418 selection for up to 7 days progressively increased the transduction efficiency to 3-fold (Hermonat, P.L. and Muzyczka, N., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6466-6470). It is expected, therefore, that efficiencies greater than 1.5% may be achieved in protocols which permit longer time periods before selection.

7.   EXAMPLE: EXPRESSION OF A BACTERIAL-
      DERIVED OXALASE GENE IN MURINE
      HEMATOPOIETIC CELLS

The examples sections detailed herein are directed to a demonstration that a bacterial-derived oxalase gene, when transferred to a mammalian (specifically, murine) host via the hematopoietic system, can lower the concentration in the circulatory system of the metabolic product oxalate. This has potential value in methods of treatment or prevention of kidney stone formation and other disorders of excess oxalate, as provided for by the present invention. The method can be divided into the following steps, for descriptive purposes only: (a) production of an AAV vector containing the oxalase gene from <u>Oxalobacter formigenes</u>,

(b) transfer of the oxalase gene to hematopoietic stem cells, (c) reconstitution of murine hosts with long-term cultured HSCs containing the bacterial-derived oxalase gene, (d) demonstration of expression of the oxalase gene in
5   blood-borne cells of the reconstituted hosts, and (e) _in vivo_ testing of the activity of the oxalase gene in preventing the build-up of oxalate in reconstituted hosts.

It is possible that there is more than one gene in the genome of O. formigenes that is involved in the
10 utilization of oxalate. Therefore, although reference will be made to the oxalase gene (singular) of O. formigenes, it is to be understood that the description herein is equally applicable to oxalase genes (plural).

15   7.1.   PRODUCTION OF AN AAV VECTOR
                        CONTAINING THE OXALASE GENE
                        FROM OXALOBACTER FORMIGENES

                  7.1.1.   ISOLATION OF THE OXALASE
                           GENE FROM O. FORMIGENES
20 The oxalase gene is isolated from Oxalobacter formigenes, a recently described anaerobic bacterium which inhabits the gastrointestinal tract of rumens as well as the colon of man (Allison, M.J., et al., 1985, Arch. Microbiol. 141:1). This bacterium utilizes oxalic acid as an energy
25 source, and thus carries an efficient oxalase gene. The advantage of the Oxalobacter formigenes oxalase lies in the fact that it has substantial activity at the physiological pH, 7.2.

                  The oxalase gene can be isolated according to
30 standard techniques (Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York; Glover, D.M. (ed.) 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford UK, Vol. I, II). Oxalobacter formigenes (ATCC No. 35274) is grown using

35

the culture conditions described by Allison et al. (Allison,
M.T., et al., supra). High molecular weight genomic DNA is
prepared from the Oxalobacter by standard techniques and a
partial digest carried out with a restriction endonuclease

5   with a 4 base pair recognition sequence, e.g., EMBO I or
SauIIIA. The partially digested DNA is size fractionated
and cloned into the pUC18 plasmid vector (available from
Bethesda Research Laboratories, Bethesda, MD).
         Selection of the recombinant plasmids expressing

10   oxalase is carried out as follows: A culture of E. coli DH5
is transformed with the pUC18 plasmid and plated on lactose
broth agar plates containing 0.05 to 0.1 M calcium oxalate.
Colonies expressing the oxalase gene have a clear zone
surrounding the bacterial growth due to the break-down and

15   utilization of the calcium oxalate. Positive colonies are
grown up, their plasmids isolated, and the foreign DNA
segment containing the oxalase gene sequenced by the di-
deoxy method of Sanger (Sanger, F., et al., 1977, Proc.
Natl. Acad. Sci. U.S.A. 74:5463) to identify the 5' and 3'

20   regions of the gene. The entire coding region of the
oxalase gene is used to construct the recombinant AAV
vector.

             7.1.2.   CONSTRUCTION OF AN ADENO-ASSOCIATED
                       VIRAL VECTOR CONTAINING THE BACTERIAL-
25   DERIVED OXALASE GENE
         The recombinant AAV vector containing the
bacterial-derived oxalase gene (AAV/Oxy vector) can be
constructed according to published procedures (Hermonat,
P.L. and Muzyczka, N., 1984, Proc. Natl. Acad. Sci. U.S.A.

30   81:6466; Tratschin, J.-D., et al., 1985, Mol. Cell. Biol.
5:3251). To generate the recombinant virus stocks, AAV/oxy
plasmid DNA and ins96/lambda-M plasmid DNA are co-
transfected into adeno-virus-2 infected HEP-2 cells using
DEAE-dextran (Hermonat and Muzyczka, supra). The

35

ins96/lambda-M plasmid is an insertion mutant which contains the <u>cap</u> and <u>lip</u> genes (required for packaging) deficient in the AAV/Oxy sequence, but is too large to be packaged in the virion (see Fig. 1). Two days post-transfection, the HEP-2

5 cells are frozen and thawed three times and passed through a 0.45 μ filter to remove cell debris. Adenovirus-2 helper virus is then inactivated by heating the virus stock at 56°C for two hours (Berns, K.I., et al., 1982, <u>in</u> Virus Persistence, Mahy, B.W.J., Mirson, A.C., and Darby, G.K.,

10 eds., Cambridge University Press, New York, pp. 249-265).

7.2. TRANSFER OF THE OXALASE GENE TO HEMATOPOIETIC STEM CELLS

7.2.1. ESTABLISHMENT OF LONG-TERM CULTURES OF BONE MARROW CELLS
15   WITH EXPANSION OF STEM CELLS

We have used bone marrow cells of <u>Mus musculus</u> (mouse) strains B10.BR/cdJ and W/W^V, aged 4-32 weeks, in order to establish long-term·cultures of hematopoietic stem. cells (HSCs). Long-term cultured bone marrow cell

20 populations have been established using a modified Dexter cell culture system (Dexter, T.M., et al., 1977, J. Cell. Physiol. 91: 335-344). Primary bone marrow cell cultures have been prepared from cells obtained by gently flushing the contents of freshly explanted femurs and tibias with a

25 Ca^{++} and Mg^{++} free Dulbecco's phosphate buffered saline. This tissue was dispersed to a cell suspension containing both single cells and small clumps, then seeded into tissue culture flasks containing Fisher's medium supplemented with horse serum and hydrocortisone succinate. After

30 approximately 3 weeks, good out-growth of an adherent cell population was observed, as shown in Figure 3. Each culture was re-seeded with freshly prepared bone marrow cells added as a single cell suspension. Within 7 days, these cultures were shown to support growth of hematopoietic stem and/or

35

progenitor cells. Cultures at this stage are shown in Figure 4 and have been carried for as long as 1 year to date. Since new cultures can be seeded with cells taken from cultures at the stage shown in Figure 4, cultures
5   selected for a specific trait can be 'rapidly' expanded.

Although the HSC is known to be present in the stromal cell layers of the long-term cultures, we have found that they also exist in significant numbers within the non-adherent populations. The use of the non-adherent cell
10 population, as opposed to the stromal layer cells, permits the expansion of the HSCs without destruction of the cultures.

To test the capacity of established long-term cultures to support the growth of the hematopoietic
15 stem/progenitor cells, we routinely determine the levels of colony forming units present after re-feedings, or the ability of the whole cell population to reconstitute lethally gamma-irradiated hosts. Non-adherent cells were carefully washed from the cultures and examined for their
20 ability to form granulocytic, erythocytic and/or monocytic (G/E/M) colonies in soft agar supplemented with L cell conditioned medium. In addition, non-adherent cell populations from the modified Dexter cultures, containing at least 300 colony forming units (G/E/M-CFUs) as measured by
25 soft agar cloning, were injected intravenously into lethally irradiated syngeneic or semi-allogeneic hosts. Successfully reconstituted hosts survived longer than 120 days, and were obtained with efficiencies approaching 100%.

30   7.2.2.   ISOLATION OF HSCs
Recent studies of Muller-Sieburg and co-workers (Muller-Sieburg, C.E., et al., 1986, Cell 44:653) indicate that a pleuripotent progenitor cell, quite likely the HSC, which is capable of producing progeny of both lymphoid and
35 myeloid lineages, can be identified in mouse bone marrow as

the Thy-1^{lo}, Ig$^-$, Ia$^-$, Lyt$^-$ cell. As few as 200 isolated cells of this phenotype can reconstitute a lethally-irradiated host. Based on these facts, a method for isolating HSCs in highly enriched or even pure form can be 5 devised.

HSCs are enriched for by fluorescent activated cell sorting (FACS). Non-adherent cell populations obtained from the long-term bone marrow cell cultures are labeled with a fluorescein isothiocyanate (FITC)-conjugated anti-10 Thy-1 monoclonal antibody (mAB). The labeled cells will be sorted and collected by FACS. Assuming that 1% of the non-adherent cells are HSCs, sorting approximately 2000 cells/sec will provide approximately 2×10^6 cells in a three hour sort.

15
7.2.3. TRANSFECTION OF HSCs USING THE AAV/OXY VECTOR SYSTEM

Populations of sorted HSCs are transfected with the bacterial oxalase gene by co-culturing the cells with the AAV/Oxy vector at a multiplicity of infection (moi) of 20 10. After 2-3 hours of co-culturing at 37°C, the mixture is centrifuged gently (700 rpm on a 6 inch rotor; approximately $125 \times g$) for 5 minutes, the supernate containing the unbound AAV/Oxy discarded, and the pelleted cells washed two times in a modified Dulbecco's phosphate buffered saline.

25

7.2.4. SELECTION OF HSCs EXPRESSING TRANSFECTED OXALASE GENE

HSCs successfully transfected with the AAV/Oxy vector and capable of expressing the bacterial oxalase gene 30 are selected prior to use in reconstitution experiments. Selection is carried out by culturing the HSC populations in the presence of a lethal concentration (1-3 mM) of potassium-oxalate (K-Ox). Calcium-free culture medium is

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supplemented with K-Ox to a concentration which kills normal
HSCs. Once selected, the HSCs are replaced on primary
stromal cells for expansion.

5   The possibility exists that the effects of K-Ox
are highly reversible following short-term incubation in the
presence of K-Ox. For instance, if only a short period of
selection is used, cells may lie dormant rather than be
killed, and thus "contaminate" the selected population. For
this reason, a second approach to the selection of
10 transfected HSCs may be used. This approach uses stromal
cells which have also been transfected with AAV/Oxy. Such
stromal cell cultures are prepared in advance of the
transfection of any HSC population. Transfected stromal
cells are selected for using medium supplemented with
15 1 mM K-Ox. This concentration of K-Ox is lethal for most
normal, non-transfected cells, yet allows long-term growth
of the cells. Thus, after selection of HSCs, the HSCs can
be propagated on stromal cells under conditions of continual
selection. This procedure has already been carried out in
studies with the AAV/Neo transfections described in Section
20 6, supra.

It is possible that the isolated and selected HSC
population is not obtained in large enough quantities to
allow further propagation after replacement on stromal
cells. In this case, total bone marrow cultures are
25 transfected and selected before enrichment for HSCs.

7.3. RECONSTITUTION OF MURINE HOSTS WITH LONG-TERM CULTURED HSCs CONTAINING THE BACTERIAL-DERIVED OXALASE GENE

30   Over the past few years, we have been developing a
number of models for bone marrow reconstitution of
sublethally and lethally irradiated hosts, especially across
the major histocompatibility barriers in fully allogeneic
combinations (Jadus, M.R. and Peck, A.B., 1984, Scand. J.
35

Immunol. 20:81). The most successful results have come from
protocols using the newborn suppressor-inducing monocyte
which can mediate suppression of any graft-versus-host
reactivity by competent cells in the donor cell population
5 (Jadus, M.R. and Peck, A.B., supra; see Section 8, infra)
and protocols using donor cell populations from the long-
term cultured bone marrow which apparently are devoid of
mature T lymphocytes. In the latter protocol, non-adherent
cells from the Dexter cultures containing at least 300
10 colony forming units (G/E/M-CFUs) were able to reconstitute
lethally gamma-irradiated syngeneic and semi-syngeneic hosts
with efficiencies approaching 100%. The efficiency of
reconstituting fully allogeneic hosts ranged between 75 -
95%. Successful reconstitution was determined first by the
15 long-term survival of the host (greater than 90 days as
opposed to lethally-irradiated, non-reconstituted hosts
which survive less than 2 weeks), secondly by the
demonstration that all blood-borne cells were of the donor
phenotype (by serotyping host lymphoid cells), and thirdly
20 by determining that the host's immunocompetence was of the
donor immune response phenotype (Figs. 7, 8).

7.3.1. HSC RECONSTITUTION OF LETHALLY-IRRADIATED INBRED MICE

At the time of HSC reconstitution, young adult
25 mice (5-6 week old C57BL/6J mice) are lethally gamma-
irradiated (950-1150 R) and placed on acid water (pH 2.0).
Approximately 4-6 hours after the irradiation, the selected
reconstituting cell populations from long-term cultured
recombinant HSCs are prepared and injected into each host
30 intravenously using the tail vein. Reconstitutions are
usually carried out using $1 \times 10^6 - 1 \times 10^7$ freshly
explanted bone marrow cells, $1 \times 10^4 - 1 \times 10^5$ cultured bone
marrow cells, or 500 - 1000 purified HSCs.

35

Following reconstitution, the mice are observed daily for signs of wasting disease or other complications. However, successful reconstitution can approach 100%.

7.3.2. HSC RECONSTITUTION OF W/W^V MICE

A second mouse system, involving mice of the W/W^V genotype, can be used. This sytem is a useful model, since relatively small numbers of recombinant HSCs are required for reconstitution. The W/W^V genotype mouse carries dominant mutations at the W locus which affects hematopoiesis. These mice have genetic defects in their HSC population; as a result, it has been reported that a single donor stem cell can repopulate the W/W^V host (Russell, E.S., 1979, Adv. Genetics 20:357; Boggs, D.R., et al., 1982, J. Clin. Invest. 70:242).

Because of the unique nature of the W/W^V genotype mice, the reconstitution protocol as described in Section 7.3.1, supra, is slightly modified. Irradiation of the hosts is no longer required. In addition, fewer cells are required for reconstitution, potentially a single isolated HSC.

7.4. DEMONSTRATION OF EXPRESSION OF THE OXALASE GENE IN BLOOD-BORNE CELLS OF THE RECONSTITUTED HOSTS

7.4.1. OXALASE GENE INCORPORATION INTO GENOMIC DNA

7.4.1.1. PREPARATION OF GENOMIC DNA

Genomic DNA is prepared from the blood-borne cells and cells residing in the lymphoid organs and tissues according to well-established procedures (Maniatis, T., et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). Briefly, mice are fasted 24 hours prior to sacrifice. The hematopoietic cells are collected, placed in liquid nitrogen, and ground to a

fine powder. The resulting cell debris is suspended in Tris-buffer containing EDTA, sodium dodecylsulfate, and proteinase K, then heated to 65°C for 16 hours. The DNA is then extracted, precipitated, collected and redissolved.

5

7.4.1.2. ENDONUCLEASE DIGESTION OF GENOMIC DNA

High molecular weight DNA is digested with restriction endonucleases under conditions described by the suppliers (Bethesda Research Laboratories, Bethesda, MD).
10 Efficiency of the digestion is analyzed by electrophoresis in 0.7% agarose gels.

7.4.1.3. DETECTION OF OXALASE GENE BY HYBRIDIZATION

Digested genomic DNA is electrophoresed through
15 0.7% agarose gels, then transferred from the gels to Zetabind nylon filters following the procedures of Southern (Southern, E., 1980, Meth. Enzymol. 69:152). The nylon filters are dried in a vacuum and can be stored for hybridization. Hybridization is carried out with a ^{32}P-
20 labeled restriction fragment containing the oxalase gene. Following hybridization, autoradiographs are produced by exposure of the hybridized filters to X-ray film. This procedure determines if the oxalase gene has successfully incorporated into the genome.
25

7.4.2. GROWTH OF CELLS IN THE PRESENCE OF POTASSIUM-OXALATE

Bone marrow cells from the reconstituted mice are used to establish long-term cultures similar to those
30 described in Section 7.2.1, supra. To determine if the oxalase gene can be expressed by the hematopoietic cells, each established culture is grown in medium supplemented

35

with potassium-oxalate (K-Ox) to a level lethal to normal
cells (1-3 mM). Growth of such cultures in the presence of
K-Ox indicates the expression of the oxalase gene.

5   7.4.3.   MEASUREMENT OF OXALASE ENZYME ACTIVITY
          Detection of the oxalase enzyme by measurements of
the enzyme activity are carried out according to the
procedures of Allison and Cook (Allison, M.J. and Cook,
H.M., 1981, Science 212:675). Oxalate degradation is
10 estimated from measurements of $^{14}C\text{-}CO_2^-$ produced from $^{14}C\text{-}$
oxalate.

          7.5.   IN VIVO TESTING OF THE ACTIVITY OF THE
                 OXALASE GENE IN PREVENTING THE BUILD-UP
                 OF OXALATE IN RECONSTITUTED HOSTS
15   The host animals reconstituted with AAV/Oxy-
transfected HSCs are tested for successful metabolism of
oxalate. The reconstituted mice are fed a diet high in
compounds converted to oxalate through normal metabolic
activity. Such compounds include ethylene glycol and
20 derivatives. This dietary challenge usually induces a state
of hyperoxalate in normal mice. Mice carrying the oxalase
gene are examined for the levels of oxalate which develop
following such dietary challenge and compared with control
animals not carrying the oxalase gene.
25
          8.   EXAMPLE: PREFERRED METHOD FOR GENERATION
               AND DETECTION OF RECOMBINANT STEM CELLS
               EXPRESSING AN OXALASE GENE
          A preferred method for the generation and
detection of recombinant stem cells expressing an oxalase
30 gene employs the procedures described infra.

35

8.1.   ISOLATION OF THE OXALASE GENE

A genomic library in pUC18 of DNA from Oxalobacter formigenes was constructed as described supra in Section 7.1.1.

5   The genomic library is screened with an oligonucleotide probe obtained by the following procedures:

Oxalase protein was isolated from a culture of O. formigenes by known techniques. Non-denaturing preparative polyacrylamide gel electrophoresis (PAGE) was used to purify
10   the 220,000 daltons molecular weight oxalase protein (comprised of two α and two β subunits). The PAGE band containing the oxalase protein was excised, and the protein was further purified by sodium dodecyl sulfate PAGE under both reducing and non-reducing conditions. A single band
15   corresponding to one oxalase subunit, and two bands corresponding to fragments of the other oxalase subunit, were observed.

The purified oxalase in the second acrylamide gel is transblotted onto PVDF paper (Immobilon, Millipore Corp.), and is subjected to amino acid sequencing to
20   determine about 15-16 amino acids of the amino-terminal oxalase sequence. On the basis of the obtained amino acid sequence, oligonucleotide probes of approximately 20 nucleotides are synthesized (Applied Biosystems DNA synthesizer) that are capable of hybridizing to the oxalase
25   gene present in the O. formigenes pUC18 genomic library. This oligonucleotide probe is used to screen the pUC18 library using known techniques of nucleic acid hybridization, to isolate the oxalase gene.

30

8.2   GENE TRANSFER INTO STEM CELLS

Stable gene transfer of the isolated oxalase gene into stem cells is accomplished by electroporation, according to the following procedure:

35

Stem cells are suspended in a buffered saline solution containing 10 micrograms DNA per milliliter, placed in a an electroporation cuvette, then exposed to the pulsating electrical current. The cuvette is a small

5   chamber fitted with aluminum electrodes. The cells are electroporated 12-25 seconds with a 250 volt current with a capacitance of 960 microFarads. The electrical pulses open small pores in the membranes of the target cells permitting DNA to enter the cytoplasm and nucleus.

10   We have observed that cell viability following the above electroporation procedure averages 40-60%. In a model transfer system using the bacterial neomycin resistance gene, efficiency of transformation approaches 10%.

15   8.3   DETECTION OF RECOMBINANT CELLS
                  EXPRESSING OXALASE ACTIVITY

The expression of oxalase activity by recombinant stem cells and their progeny can be demonstrated by detection of CO_2 release due to substrate (oxalate) degradation. CO_2 is released into the atmosphere by

20   acidification and, if radioactive (e.g., ^{14}C-labeled), will darken vicinal photographic film. The procedure is the following:

(i)   Wells of a microtiter plate are loaded with 200 μl of cell suspension (or oxalase-containing solution) plus

25   10 μl 20 mM [^{14}C]-sodium oxalate dissolved in cell suspension medium (each well should contain about 1 μl).

(ii)   Mix and incubate at 37°C for four hours.

(iii)   Rapidly set up ASA 1000 film exposure (less than two

30   minutes).

(iv)   Acidify wells by adding 10 μl 2 M HCl to each well; mix.

35

(v)   In a hood in a darkroom, cover the wells with film (light sensitive side to wells). Use a glass or plastic plate on top to hold the film in place.

(vi)   Let the film expose for about four hours, then develop the film. The location of region(s) darkened by $[^{14}C]$- CO_2 can be matched to the location of the microtiter plate well(s) containing specific metabolic activity.

It should be noted that the procedures described _supra_ in Sections 8.1 and 8.2 can potentially be adapted for use with many different metabolases. The procedures described in Section 8.3 can be used in the detection of metabolase activities which result in the production of CO_2.

9. EXAMPLE: PREFERRED METHOD FOR ISOLATION OF A URICASE GENE

In a preferred embodiment, a porcine genomic library is screened for a uricase gene by hybridization to the following oligonucleotide probe (encoding amino acid residue numbers 13-21 of porcine urate oxidase (Lee, C.C., et al., 1988, Science 239:1288-1291), shown below the DNA sequence):

```
5'                                                      3'
   ACT  GGC  TAT  GGG  AAG  GAT  ATG  ATA  AAA
   Thr  Gly  Tyr  Gly  Lys  Asp  Met  Ile  Lys
```

A porcine liver genomic library was constructed by digestion of porcine liver DNA with the restriction enzymes _MboI_ and _Sau3A_, followed by ligation into pUC18. The library is screened by known techniques for hybridization to the (^{32}P-labeled) oligonucleotide probe shown above.

10.   EXAMPLE: SUCCESSFUL SEMI-ALLOGENEIC AND ALLOGENEIC
       BONE MARROW RECONSTITUTION OF LETHALLY IRRADIATED
       ADULT MICE MEDIATED BY NEONATAL SPLEEN CELLS

           Spleens of fetal/newborn mice less than 3-4 days
of age contain a naturally occurring cell population capable
5 of suppressing T-dependent and T-independent immune
responses of third-party adult cells both in vitro and in
vivo. This study was undertaken to determine if spleen
cells from newborn mice were capable of preventing graft-
versus-host (GVH) disease in lethally irradiated hosts
10 reconstituted with either semiallogeneic or allogeneic bone
marrow cells. Earlier studies (Jadus, M. and Peck, A.B.,
1984, Scand. J. Immunol. 20:81) have shown that lethal GVH
disease induced by injecting semi-allogeneic or allogeneic T
lymphocytes into sublethally-irradiated hosts was inhibited
15 if the T cells had been co-cultured 24 hours in the presence
of isolated newborn monocyte populations. We have utilized
newborn spleen cells to prevent acute GVH disease in
lethally-irradiated adult hosts reconstituted with semi-
allogeneic or even allogeneic bone marrow cells.
20 Pretreatment of reconstituting cell populations with newborn
spleen cells reduced the incidence of GVH disease from 100
to 20% in semi-allogeneic and from 100 to 40% in allogeneic
combinations. Long-term surviving reconstituted hosts
proved immunologically unresponsive to both donor and host
25 histocompatibility antigens, yet possessed a fully chimeric
lymphoid system responsive to T and B cell mitogens as well
as unrelated third-party alloantigens.

           10.1.   MATERIALS AND METHODS

30

           10.1.1.   ANIMALS
           Mice used in this study were bred and maintained
in the animal facility located in the Department of
Pathology, University of Florida, Gainesville, Florida.

35

They included AKR/J, C57BL/6J, C57BL/10Sn, B10.BR/cd, B10.Q, B10.RIII(71NS), B10.S, CBA/H, CBA/J, DBA/2J, B10.SAA48, B10.BUA16 and (C57BL/10Sn x B10.BR/cd)F_1 hybrids. Donor and host mice were age and sex matched for all bone marrow reconstitution experiments. Mice used in reconstitution experiments ranged from 6-10 weeks of age while mice used for functional assays ranged from 6-24 weeks of age.

10.1.2. ANTISERA

Alloantisera D.32 (anti-H-2D^k) and K.333 (antiH-2K^b), provided by Dr. E.K. Wakeland (Department of Pathology, University of Florida, Gainesville, Florida), as well as 15-3-1S (anti-H-2K^k) and 28-13-3S (anti-H-2K^b), obtained from the American Type Culture Collection, Rockville, MD., were used to serotype for the histocompatibility antigens expressed on spleen cells of the chimeric animals.

10.1.3. SPLEEN CELL PREPARATIONS

Single-cell suspensions of newborn and adult splenocytes were prepared by gently pressing freshly explanted tissues through wire mesh screens, and washing with phosphate-buffered saline (PBS). For mixed leukocyte culture (MLC) reactions, the red blood cells were lysed with 0.84% ammonium chloride. The leukocytes were then washed once and resuspended in PBS to the appropriate concentrations.

10.1.4. BONE MARROW CELL PREPARATIONS

Adult bone marrow cells were prepared by cutting the epiphyses from freshly explanted femurs and tibias and then flushing the contents from the lumens with PBS using a 27 gauge needle and syringe. The tissue was dispersed to a single cell suspension using gentle pipetting.

10.1.5. BONE MARROW RECONSTITUTION OF LETHALLY IRRADIATED HOSTS

Newborn splenocytes were cultured at a concentration of 6×10^6 per plate for 24 hours at 37°C and 5% CO_2 in 3 ml medium per 35 cm^2 plate. Eagles' high amino acid (EHAA) medium was used, supplemented to 0.5% normal mouse serum (Peck, A.B. and Click, R.E., 1973, Eur. J. Immunol. 3:385). This culture period permitted suppressor factors to be produced and secreted. Freshly explanted adult donor bone marrow cells (30 x $1Q^6$ per plate) plus whole spleen cells (30 x 10^6 per platë) were then either cultured alone or co-cultured with the newborn splenocytes for an additional 24 hours. The cells were collected, washed and resuspended at a concentration of 20 x 10^6 cells per 0.2 ml. Lethally gamma-irradiated (970 Rad) recipient mice received 0.2 ml per mouse, by intravenous injection via the tail vein, within 4 hours after irradiation.

Reconstituted animals were usually placed in laminar flow hoods and given water containing 10 mg/L polymyxin B plus 100 mg/L neomycin. In a few experiments, the mice were maintained under normal colony conditions and given acidified water to drink, but no differences in survival time or rates were observed. At various time points, recipient mice were killed for histological and functional studies. Single cell suspensions of splenocytes were prepared as described above and tested for functional reactivities in mitogen stimulation and mixed leukocyte culture assays.

10.1.6. LYMPHOCYTE CULTURES

Mixed leukocyte culture (MLC) assays were performed as previously described (Peck, A.B. and Bach, F.H., 1973, J. Immunol. Methods 3:147), and consisted of 0.5 x 10^6 splenic leukocytes co-cultured in flat bottom plates (#3596, Costar, Cambridge, MA) with an equal number of

gamma-irradiated (2500 Rad) stimulating splenic leukocytes
in 0.2 ml EHAA. Cells were pulsed with 1.0 mCi of tritiated
thymidine at various time points, harvested 8 hours later,
and ^{3}H-uptake measured using standard scintillation

5 procedures. Mitogenic responses were measured in a similar
manner following stimulation with either 8 ug concanavalin A
(Con A) (Pharmacia Fine Chemicals, Piscataway, NJ) or 25 ug
lipopolysaccharide (LPS) (S. typhimurium, Sigma Chemical
Co., St. Louis, MO). Data in Figures 7 and 8 are reported

10 as the means of triplicate cultures minus background.
Standard deviations of the means are indicated by the
vertical line drawn through each data point.

10.1.7. COMPLEMENT-DEPENDENT CYTOTOXIC ASSAY

15   Leukocytes were serotyped using a two-step
cytotoxicity assay. Spleen cells at 1.0 x 10^6 cells per ml
were incubated with appropriate antisera for 45 minutes at
4°C. The cells were then washed, resuspended in rabbit
complement (Accurate Chemical and Scientific Corp.,

20 Hickesville, NY) and incubated for 45 minutes at 37°C. All
tests were carried out in RPMI 1640 medium (GIBCO, Grand
Island, NY). Cell viability was assessed by trypan blue dye
exclusion.

10.1.8. HISTOLOGICAL EXAMINATIONS

25   Freshly removed organs and skin tissue were placed
in 10% formalin for 18-24 hours, then placed in 80% ethanol.
Tissues were embedded in paraffin, sectioned, and stained
with hematoxylin-eosin dye.

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10.2.　RESULTS

10.2.1.　NEWBORN SPLEEN CELLS PREVENT GRAFT VS. HOST DISEASE IN HOSTS ENGRAFTED WITH SEMI-ALLOGENEIC BONE MARROW

5　　　　Lethally gamma-irradiated (C57BL/10 x B10.BR/cd)F_1 hosts were reconstituted with B10.BR/cd bone marrow cells with and without CBA/J newborn spleen cells. Host survival is shown in Table VII.

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TABLE VII

SUPPRESSION OF LETHAL GVH DISEASE BY NEWBORN SPLEEN CELLS IN LETHALLY-IRRADIATED $(C57BL/10 \times B10.BR/cd)F_1$ HOSTS RECONSTITUTED WITH SEMI-ALLOGENEIC ADULT B10.BR/cd BONE MARROW CELLS

Reconstituting Cell Population	Ratio of Adult to Newborn Cells	Survival Times (days)[1]	% Long-Term Survivals	Mean Survival Times (days)
Experiment 1:				
None	-	9,10,10	0	10
B10.BR/cd adult bone marrow cells	-	12,14,15	0	14
B10.BR/cd adult bone marrow cells + CBA/J newborn spleen cells	10:1	8,>60,>120, >170,>190	80	>112
Experiment 2:				
B10.BR/cd adult bone marrow cells	-	8,8,8,8, 8,8,13,16	0	10
B10.BR/cd adult bone marrow cells + CBA/J newborn spleen cells	10:1	8,8,>35,>55, >103,>103,>130, >130,>130,>130	80	>83

[1] The symbol > indicates that the host animal, although healthy, was killed for functional studies on that day, or presently still alive.

As shown in Table VII, all irradiation control
mice injected with PBS died within 15 days. Likewise,
control animals injected with B10.BR/cd bone marrow cells
cultured for 24 hours alone, died within 16 days. However,
5 80% of hosts reconstituted with B10.BR/cd bone marrow cells
co-cultured for 24 hours with CBA/J newborn suppressor cells
were long-term survivors, living greater than 60 days (Fig.
5). Long-term surviving hosts have been followed for as
long as 6 months after reconstitution before being killed
10 for functional studies. Similar data have been obtained
with this protocol using several genetic combinations.

10.2.2. NEWBORN SPLEEN CELLS PREVENT GRAFT VS. HOST DISEASE IN HOSTS ENGRAFTED WITH ALLOGENEIC BONE MARROW

15 In a second set of experiments, lethally gamma-
irradiated C57BL/6 hosts were reconstituted with B10.BR/cd
bone marrow cells with and without CBA/J newborn spleen
cells. Host survival is shown in Table VIII.

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30

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TABLE VIII

SUPPRESSION OF LETHAL GVH DISEASE BY NEWBORN SPLEEN CELLS IN LETHALLY-IRRADIATED C57BL/6J HOSTS RECONSTITUTED WITH ALLOGENEIC ADULT B10.BR/cd BONE MARROW CELLS

Reconstituting Cell Population	Ratio of Adult to Newborn Cells	Survival Times (days) [1]	% Long-Term Survivals	Mean Survival Times (days)
Expt. 1				
None	–	3,3,4,10,10, 10,11,11,11, 12,12,13,13, 13,14	0	10
C57BL/6J adult bone marrow cells	–	>92,>92,>92, >92,>92,>92, >92,>92,>92, >92	100	>92
Expt. 2:				
None	–	5,6,11,12, 13,15	0	10
C57BL/6J adult bone marrow cells	–	16,>77,>77 >77,>87,>87	83	>63
B10.BR/cd adult bone marrow cells	–	9,11,11	0	10
B10.BR/cd adult bone marrow cells + CBA/J newborn spleen cells	10:1	8,9,11,>21, >77,>87	50	32

Expt. 3:

B10.BR/cd adult bone marrow cells	-	7,7,9,9	0	8
B10.BR/cd adult bone marrow cells + CBA/J newborn spleen cells	10:1	7,7,9,>90, >90,>90	50	>49

Expt. 4:

B10.BR/cd adult bone marrow cells	-	7,11,11,15, 19,27	0	15
B10.BR/cd adult bone marrow cells + CBA/J newborn spleen cells	10:1	17,20,>15, >33,>42,>55, >120	71	>43

[1]The symbol > indicates that the host animal, although healthy, was killed for functional studies on that day, or is presently still alive.

     As shown in Table VIII, all irradiation control animals and animals injected with untreated B10.BR/cd bone marrow cells died within 19 days, with one animal dying at 27 days. Under the experimental and environmental conditions used here, 90-100% of recipients receiving syngeneic bone marrow survived, whereas 55-60% of hosts reconstituted with B10.BR/cd bone marrow co-cultured with CBA/J newborn suppressor cells were long-term survivors

(Fig. 5). These data indicate that newborn spleen cells can mediate successful development of fully allogeneic engrafted hosts.

We have thus shown that lethally-irradiated hosts can be successfully reconstituted with semi-allogeneic (80%-90% long-term survival) or allogeneic (55%-60% long-term survival) bone marrow if the donor cells are first co-cultured 24 hours with newborn spleen cells (Tables VII and VIII).

10.2.3. HISTOLOGICAL EXAMINATION OF EXPERIMENTAL MICE

To confirm that death of experimental animals following reconstitution resulted from GVH disease and not irradiation per se, histological studies were done. Histological pictures of skin biopsies as well as liver and spleen sections are presented in Figure 6. Irradiation control mice showed fairly normal skin biopsies (Fig. 6a) with the exception of noticeable loss of fat cells between connective tissue of the dermis and muscle. Similarly, liver histology appeared normal (Fig. 6b). However, spleens from irradiation control mice showed severe generalized cytopenia in both white and red pulp, a collapsed architecture disrupting the white and red pulp areas, hemosiderin, and markedly visible connective tissue septae (Fig. 6c). Experimental mice which were successfully reconstituted with bone marrow pretreated with newborn suppressor cells displayed normal skin (Fig. 6d), liver (Fig. 6e), and spleens (Fig. 6f). Occasionally, slight leukocytic cell infiltration could be seen around portal tracts of the livers.

In contrast, experimental mice which failed to thrive following bone marrow reconstitution showed signs of severe GVH disease: emaciation, exaggerated hunched appearance, patches of hair loss, sluffing of the tails,

development of diarrhea, and hypothermia. Skin biopsies
revealed an atrophic epidermal layer and a hyperkeratotic
surface, as well as a loss of skin appendages (Fig. 6g).
Upon closer examination, there were focal lymphocyte
5 .infiltrates within the surface epithelium, and increased
mononuclear cell numbers in and around blood vessels and the
remains of skin appendages. The livers (Fig. 6h) contained
marked inflammatory lesions: inflammatory cell infiltrates
were evident within the portal tracts whereas focal cell
10 infiltrates were present in the sinusoids of the lobules and
associated with patchy hepatocyte necrosis. A few livers
showed fibrosis with inflammation and necrosis at the
periphery of fibrotic regions. Spleens from these mice had
expanded and delineated white pulp regions which contained
15 cells with morphologic characteristics of blast
transformation. At the time of severe cachexia, the white
pulp showed lymphocyte depletion. The red pulp had
collapsed, and Russell bodies, septae and hemosiderin were
visible.

20   The histological examination of mice which had
died or been killed for functional testing clearly revealed
several points: 1) lethal irradiation caused complete
destruction of the lymphoid compartment of the host, 2)
hosts reconstituted with histoincompatible bone marrow
25 showed successful engraftment but subsequent development of
severe organ and tissue lesions characteristic of classic
GVH disease, and 3) hosts successfully reconstituted with
histoincompatible bone marrow pretreated with newborn spleen
cells showed normal lymphoid and tissue histology. Thus,
30 pretreatment of histoincompatible donor bone marrow cells
with newborn spleen cells prevented development of lethal
GVH disease prevalent in host animals engrafted with
untreated bone marrow cells.

35

10.2.4.   FULL CHIMERISM IN LONG-TERM SURVIVORS

Data presented in Table IX show that the surviving semi-allogeneic and allogeneic bone marrow reconstituted hosts were fully chimeric. The leukocyte populations residing in the spleens of (C57BL/10 x B10.BR/cd)F_1 hosts reconstituted with B10.BR/cd, as well as the C57BL/6J hosts reconstituted with B10.BR/cd, serotyped positive for H-2^k but negative for H-2^b.

TABLE IX

SEROTYPING OF SPLENOCYTES FROM LONG-TERM SURVIVING RECONSTITUTED MICE

Set #	Spleen Cell Population Tested	Cytotoxicity with[1]		
		anti-H-2^k + C'	anti-H-2^b + C'	C'
1a	B10/BR/cd	80%	25%	25%
	(C57BL/10 x B10.BR/cd)F$_1$ reconstituted with B10.BR bone marrow cells + CBA/J newborn splenocytes	88%	10%	15%
1b	B10/BR/cd	85%	21%	–
	C57BL/J	16%	99%	–
	(C57BL/10 x B10.BR/cd)F$_1$ reconstituted with B10.BR bone marrow cells + CBA/J newborn splenocytes	96%	13%	–
2a	C57BL/10	25%	88%	20%
	C57BL/6J reconstituted with C57BL/6J bone marrow cells	15%	80%	15%
	C57BL/6J reconstituted with B10.BR/cd bone marrow cells + CBA/J newborn splenocytes	80%	15%	15%

2b	B10.BR/cd	83%	13%	–
	C57BL/6J reconstituted with C57BL/6J bone marrow cells	8%	99%	–
	C57BL/6J reconstituted with B10.BR/cd bone marrow cells + CBA/J newborn splenocytes	96%	37%	–

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[1]Lymphocytotoxicity determined in a two step C′-dependent cytotoxicity assay.

10.2.5. FUNCTIONAL STUDIES ON SPLENOCYTES FROM LONG-TERM SURVIVORS

Semi-allogeneic and allogeneic bone marrow reconstituted hosts were killed at various times and their splenocytes assayed for immune reactivity to mitogenic stimulation and MLC reactivity (Figs. 7 and 8). Cells from $(C57BL/10 \times B10.BR/cd)F_1$ recipients reconstituted with B10.BR/cd cells responded strongly to Con A and LPS stimulation, thus demonstrating immunocompetence of the T cell and B cell compartments at 60 days (Fig. 7A). In addition, they responded to third-party alloantigens, e.g., DBA/2J ($H-2^d$, Mls^a) and B10.SAA48 ($H-2^{w3}$), while failing to respond to B10.BR/cd and C57BL/10 stimulator cells (Fig. 7B). In addition, these cells responded against B10.RIII(71NS) ($H-2^r$), B10.S ($H-2^s$) and B10.BUA16 ($H-2^{w22}$) stimulators. These data indicate that the cells residing in the $(C57BL/10 \times B10.BR/cd)F_1$ chimeric hosts were tolerant to

both donor and recipient histocompatiblity antigens but not to third party alloantigens. This substantiates the immunocompetence of the T cell compartment of these hosts.

Similarly, C57BL/6 hosts reconstituted with
5   B10.BR/cd bone marrow cells were assayed for mitogenic stimulation and MLC reactivity. Figure 8 shows data for mice sacrificed on day 90. The cells proved unresponsive to CBA/H and C57BL/6 cells, indicating a tolerance to both donor and recipient haplotypes. In contrast, the cells were
10   reactive to both LPS and histocompatibility alloantigens, e.g., expressed by B10.Q ($H-2^q$), AKR/J ($H-2^k$, Mls^d) and DBA/2J ($H-2^d$, Mls^a). Thus, these reconstituted hosts also exhibited immunocompetence of the T and B cell compartments.

One interesting and unexpected result was the
15   response of the chimeric (C57BL/10 x B10.BR/cd)F_1 hosts toward adult CBA/J cells. Although syngeneic with the newborn suppressor-inducer population, CBA/J cells stimulated cells of the reconstituted hosts. This lack of tolerance may result from an insufficient quantity of CBA/J
20   alloantigens present during the development of the chimeric immune system, or alternatively, tolerance toward non-MHC alloantigens, e.g. Mls, may never develop in such a protocol.

Thus, functional studies using spleen cells from
25   reconstituted chimeric hosts surviving greater than 60 days showed strong proliferation in response to both T and B cell mitogens as well as alloantigens on third party cells (Figs. 7 and 8). In contrast, these chimeric mice proved tolerant to both donor and host histocompatibility antigens.
30   Serotyping confirmed that the hosts were fully chimeric with all the splenocytes expressing donor histocompatibility antigens (Table IX). These results are in marked contrast to those reported by Jadus and Peck (1981, Scand. J. Immunol. 20:81). In that study, cells from sublethally-
35   irradiated, T cell engraftment hosts examined at day 60

post-engraftment remained tolerant to host cells but
responded to donor and third-party cells. Furthermore, at
day 60 all of the splenocytes serotyped as host cells.

5   11.   EXAMPLE: PRODUCTION OF MONOCLONAL
                ANTIBODIES TO OXALASE

        Monoclonal antibodies to oxalase polypeptides
purified by polyacrylamide gel electrophoresis (PAGE) (as
described supra, Section 8.1) are produced by procedures
known in the art (see, e.g., Kohler and Milstein, 1975,
10   Nature 256:495-497). RIBA adjuvant plus the oxalase-
containing acrylamide bands were injected as immunogen into
a BALB/c mouse. Three injections were done over a period of
three months. Spleen cells from the immunized mouse are
then fused with SP2/0 mouse myeloma, to produce hybridoma
15   cells secreting monoclonal antibody. The hybridoma cells
are screened for the production of anti-oxalase antibody by
ELISA (enzyme-linked immunosorbent assay) using purified
oxalase as antigen.

20   It is apparent that many modifications and
variations of this invention as hereinabove set forth may be
made without departing from the spirit and scope thereof.
The specific embodiments described are given by way of
example only and the invention is limited only by the terms
25   of the appended claims.

30

35

WHAT IS CLAIMED IS:

        1. A recombinant stem cell comprising a stem cell
in which a heterologous gene sequence which encodes an
5   enzyme capable of altering or degrading a metabolite is
stably incorporated, which recombinant stem cell is capable
of generating progeny cells which express the heterologous
gene.

10   2. The recombinant stem cell of claim 1 in which
the stem cell is a hematopoietic stem cell.

        3. The recombinant stem cell of claim 1 in which
the stem cell is an epithelial stem cell.

15
        4. The epithelial stem cell of claim 3 in which
the stem cell is derived from the skin.

        5. The epithelial stem cell of claim 3 in which
20 the stem cell is derived from the lining of the gut.

        6. The recombinant stem cell of claim 1 in which
the stem cell is derived from embryonic heart muscle.

25   7. The recombinant stem cell of claim 1, 2, 3, or
6 in which the enzyme is an oxalase.

        8. The recombinant stem cell of claim 7 in which
the oxalase is an oxalate oxidase.

30
        9. The recombinant stem cell of claim 7 in which
the oxalase is an oxalate decarboxylase.

        10. The recombinant stem cell of claim 1 in which
35 the heterologous gene sequence is derived from a bacterium.

11.   The recombinant stem cell of claim 9 in which the oxalate decarboxylase is encoded by a heterologous gene sequence derived from a bacterium.

5   12.   The recombinant stem cell of claim 11 in which the bacterium is <u>Oxalobacter</u> <u>formigenes</u>.

13.   The recombinant stem cell of claim 1, 2, 3, or 6 in which the enzyme comprises a uricase.

10

14.   The recombinant stem cell of claim 1 in which the heterologous gene sequence is derived from a mammal.

15.   The recombinant stem cell of claim 13 in
15 which the heterologous gene sequence is derived from a mammal.

16.   The recombinant stem cell of claim 15 in which the heterologous gene sequence is derived from porcine
20 liver.

17.   The recombinant stem cell of claim 1, 2, 3, or 6 into which the heterologous gene sequence is introduced by electroporation.

25

18.   The recombinant stem cell of claim 1, 2, 3, or 6 into which the heterologous gene sequence is introduced by transfection of DNA comprising the heterologous gene sequence.

30

19.   The recombinant stem cell of claim 1, 2, 3, or 6 into which the heterologous gene sequence is introduced by use of a recombinant vector comprising the heterologous gene sequence.

35

20.   The recombinant stem cell of claim 19 in
which the vector is a virus.

21.   The recombinant stem cell of claim 20 in
5 which the virus is a retrovirus.

22.   The recombinant stem cell of claim 20 in
which the virus is a papovavirus.

23.   The recombinant stem cell of claim 20 in
10 which the virus is a vaccinia virus.

24.   The recombinant stem cell of claim 20 in
which the virus is a parvovirus.

15

25.   The recombinant stem cell of claim 24 in
which the parvovirus is an adeno-associated virus.

26.   The recombinant stem cell of claim 1, 2, 3 or
20 6 in which the metabolite is selected from the group
consisting of oxalate, uric acid, a phenylketone,
phenylpyruvic acid, phenylethylamine, a porphyrin, delta-
amino levulinic acid, testosterone, and cholesterol.

25   27.   A recombinant epithelial cell comprising an
epithelial cell in which a heterologous gene sequence which
encodes an antibody molecule, or a fragment thereof, is
incorporated, which recombinant epithelial cell is capable
of generating progeny cells which express the antibody
30 molecule or the binding domain thereof.

28.   The recombinant epithelial cell of claim 27
in which the antibody molecule recognizes an epitope of a
pathogenic microorganism.

35

29.   The recombinant epithelial cell of claim 28 in which the pathogenic microorganism is a bacterium.

30.   The recombinant epithelial cell of claim 29 in which the bacterium is a Pseudomonas.

31.   A recombinant nucleic acid vector comprising a heterologous gene sequence which encodes an oxalase.

32.   The recombinant vector of claim 31 in which the heterologous gene sequence is derived from a bacterium.

33.   The recombinant vector of claim 32 in which the bacterium is Oxalobacter formigenes.

34.   A recombinant nucleic acid vector comprising a heterologous gene sequence which encodes a uricase.

35.   A recombinant vector for stably transferring to a stem cell a heterologous gene sequence which is heritable and expressible by progeny of the stem cell, comprising a heterologous gene sequence which encodes an enzyme capable of altering or degrading a metabolite.

36.   The recombinant vector of claim 35 in which the enzyme is an oxalase.

37.   The recombinant vector of claim 36 in which the oxalase is an oxalate decarboxylase.

38.   The recombinant vector of claim 35 in which the heterologous gene sequence is derived from a bacterium.

39.   The recombinant vector of claim 37 in which the heterologous gene sequence is derived from a bacterium.

40. The recombinant vector of claim 39 in which the bacterium is <u>Oxalobacter</u> <u>formigenes</u>.

41. The recombinant vector of claim 40 in which
5 the vector is an adeno-associated virus.

42. The recombinant vector of claim 35 in which the vector is an adeno-associated virus.

10 43. The recombinant vector of claim 35 in which the enzyme is a uricase.

44. The recombinant vector of claim 35 in which the heterologous gene sequence is derived from a mammal.

15
45. The recombinant vector of claim 43 in which the heterologous gene sequence is derived from a mammal.

46. The recombinant vector of claim 45 in which
20 the uricase is encoded by a heterologous gene sequence derived from porcine liver.

47. The recombinant vector of claim 46 in which the vector is an adeno-associated virus.

25
48. The recombinant vector of claim 35 in which the vector is a virus.

49. The recombinant vector of claim 48 in which
30 the virus is a retrovirus.

50. The recombinant vector of claim 48 in which the virus is a papovavirus.

35

51.   The recombinant vector of claim 48 in which
the virus is a vaccinia virus.

52.   The recombinant vector of claim 48 in which
the virus is a parvovirus.

53.   The recombinant vector of claim 35, 42 or 48
in which the metabolite is selected from the group
consisting of oxalate, uric acid, a phenylketone,
phenylpyruvic acid, phenylethylamine, a porphyrin, delta-
amino levulinic acid, testosterone, and cholesterol.

54.   A method for the treatment or prevention of a
disorder resulting from an increased concentration or
accumulation of a metabolite in a host, comprising
introducing into or onto the host a recombinant stem cell,
or its progeny, in which stem cell or progeny is
incorporated a heterologous gene sequence encoding an enzyme
capable of altering or degrading the metabolite, which
heterologous gene sequence is expressed as a functional
enzyme in the host and which enzyme alters or degrades the
metabolite in the host.

55.   The method according to claim 54 in which the
metabolite is oxalate.

56.   The method according to claim 55 in which the
enzyme is an oxalase.

57.   The method according to claim 56 in which the
enzyme is an oxalate oxidase.

58.   The method according to claim 56 in which the
oxalase is an oxalate decarboxylase.

59.   The method according to claim 56 in which the oxalase is encoded by a heterologous gene derived from a bacterium.

5   60.   The method according to claim 58 in which the oxalate decarboxylase is encoded by a heterologous gene derived from a bacterium.

61.   The method according to claim 60 in which the
10   bacterium is Oxalobacter formigenes.

62.   The method according to claim 55 in which the disorder is kidney stone formation.

15   63.   The method according to claim 55 in which the disorder is hyperoxaluria.

64.   The method according to claim 55 in which the disorder is oxalosis.

20
65.   The method according to claim 55 in which the disorder is renal failure.

66.   The method according to claim 55 in which the
25   disorder is induced by ethylene glycol ingestion.

67.   The method according to claim 55 in which the disorder is induced by xylitol administration.

30   68.   The method according to claim 54 in which the metabolite is uric acid.

69.   The method according to claim 68 in which the enzyme is a uricase.

35

70. The method according to claim 69 in which the uricase is encoded by a heterologous gene sequence derived from a mammal.

5   71. The method according to claim 70 in which the uricase is encoded by a heterologous gene sequence derived from porcine liver.

72. The method according to claim 68 in which the
10 disorder is uric acid nephrolithiasis.

73. The method according to claim 72 in which the disorder is kidney stone formation.

15   74. The method according to claim 68 in which the disorder is gout.

75. The method according to claim 68 in which the disorder is renal failure.

20

76. The method according to claim 54 in which the recombinant stem cell, or its progeny, contain multiple heterologous gene sequences encoding enzymes capable of altering or degrading metabolites.

25
77. The method according to claim 76 in which the heterologous gene sequences are an oxalase gene and a uricase gene.

30   78. The method according to claim 54 in which the metabolite is selected from the group consisting of a phenylketone, phenylpyruvic acid, phenylethylamine, a porphyrin, delta-amino levulinic acid, testosterone, and cholesterol.

35

79. A method for the treatment or prevention of a
disorder caused by a pathogenic microorganism comprising
introducing into or onto the host a recombinant stem cell,
or its progeny, in which stem cell or progeny is
5 incorporated a heterologous gene sequence encoding an
antibody molecule, or a fragment thereof, which heterologous
gene sequence is expressed as an antibody molecule or the
binding domain thereof and which antibody molecule or
binding domain binds to an epitope of the pathogenic
10 microorganism.

80. The method according to claim 79 in which the
epithelial stem cell progeny cell is a keratinocyte.

15 81. The method according to claim 79 in which the
pathogenic microorganism is a bacterium.

82. The method according to claim 79 in which the
pathogenic microorganism is a virus.

20
83. The method according to claim 81 in which the
recombinant cell is applied to a burn.

84. The method according to claim 83 in which the
bacterium is a Pseudomonas.
25

85. A method for the treatment or prevention of a
disorder due to an increased concentration or accumulation
of a metabolite in a host, comprising introducing into or
onto the host a synthetic molecule which binds the
30 metabolite, said synthetic molecule being predicted by
molecular modelling to have a similar conformation to an
enzyme which alters or degrades the metabolite.

35

86. The method according to claim 85 in which the metabolite is oxalate.

87. The method according to claim 85 in which the metabolite is uric acid.

88. The method according to claim 85 in which the metabolite is selected from the group consisting of a phenylketone, phenylpyruvic acid, phenylethylamine, a porphyrin, delta-amino levulinic acid, testosterone, and cholesterol.

89. The method according to claim 85 in which the synthetic molecule is also capable of altering or degrading the metabolite.

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FIG. 1
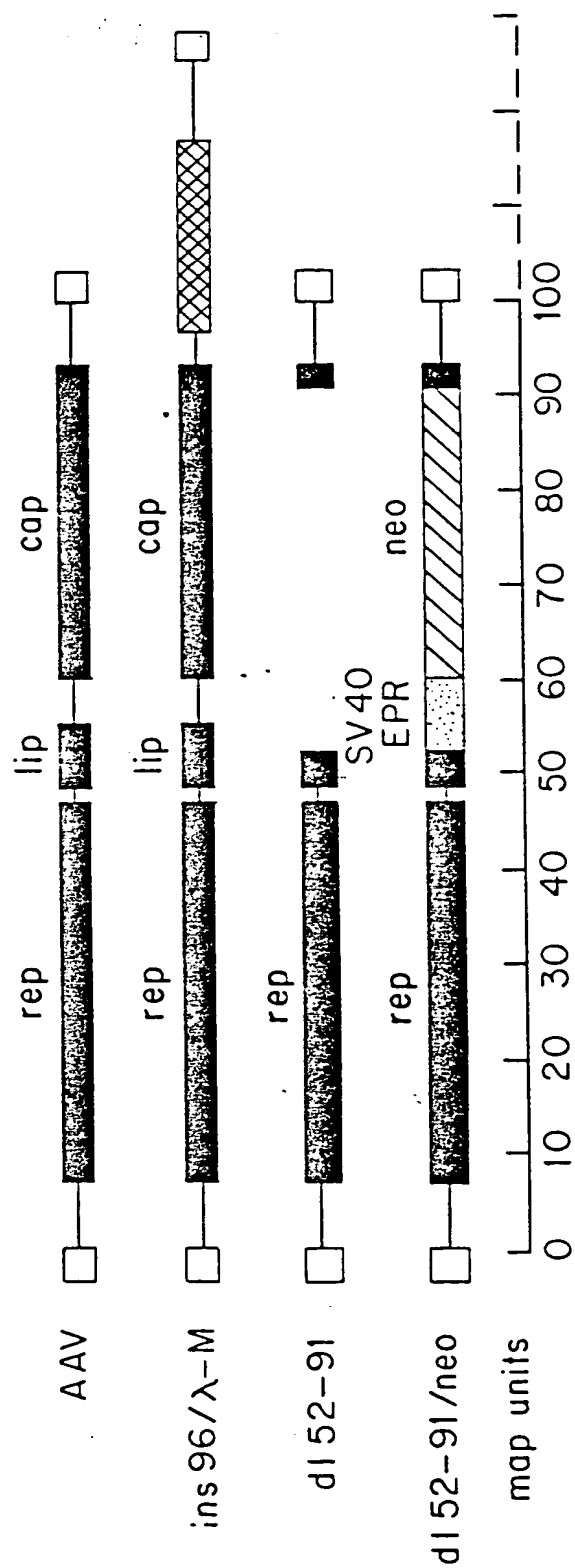
Fig. 2a
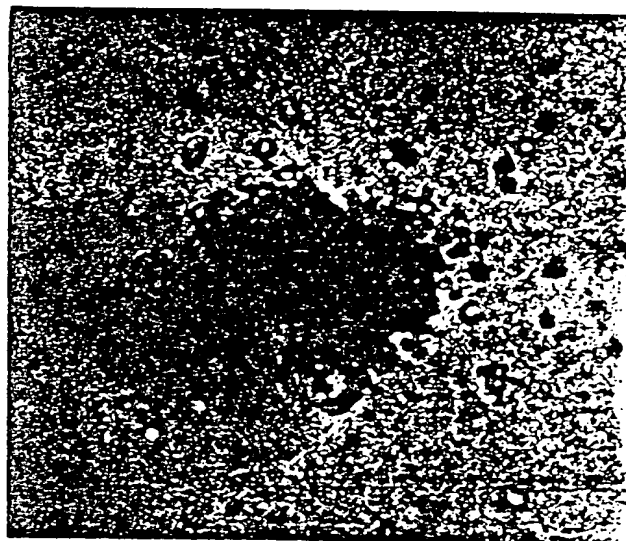
Fig. 2 b
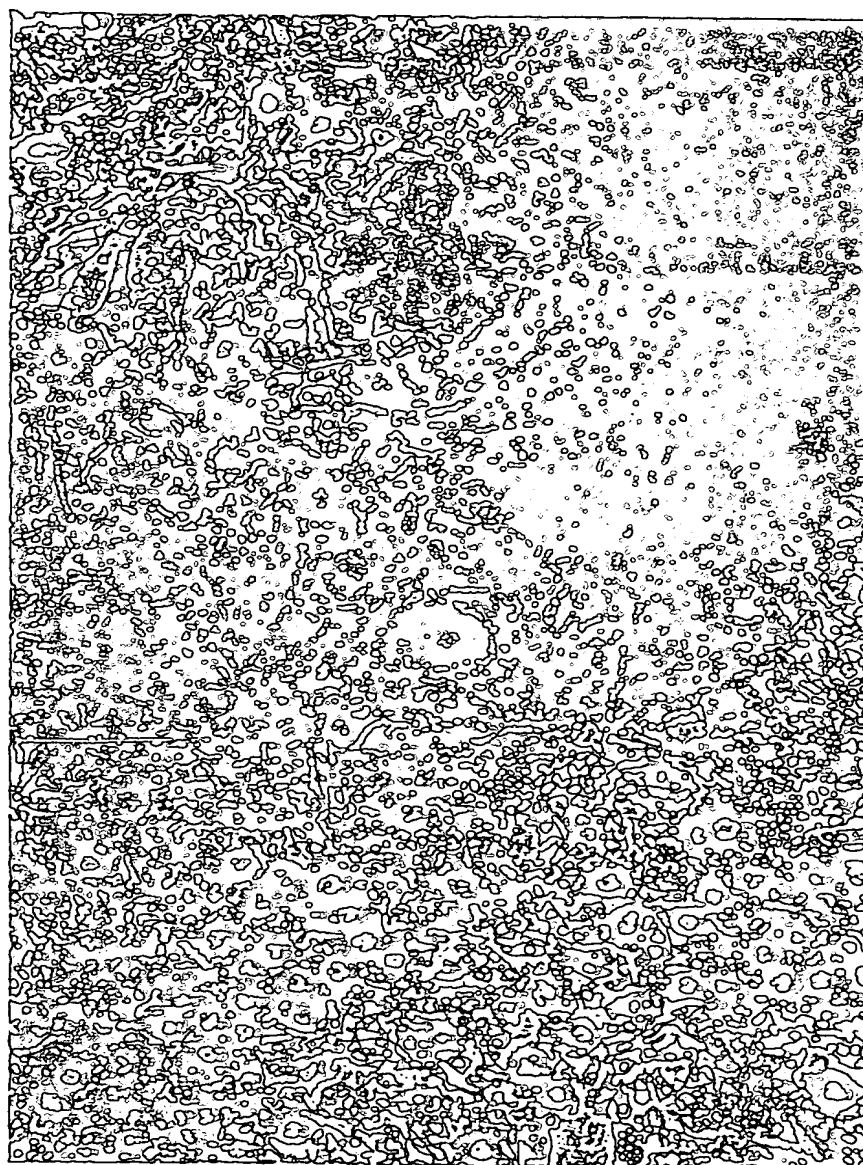
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Fig. 3

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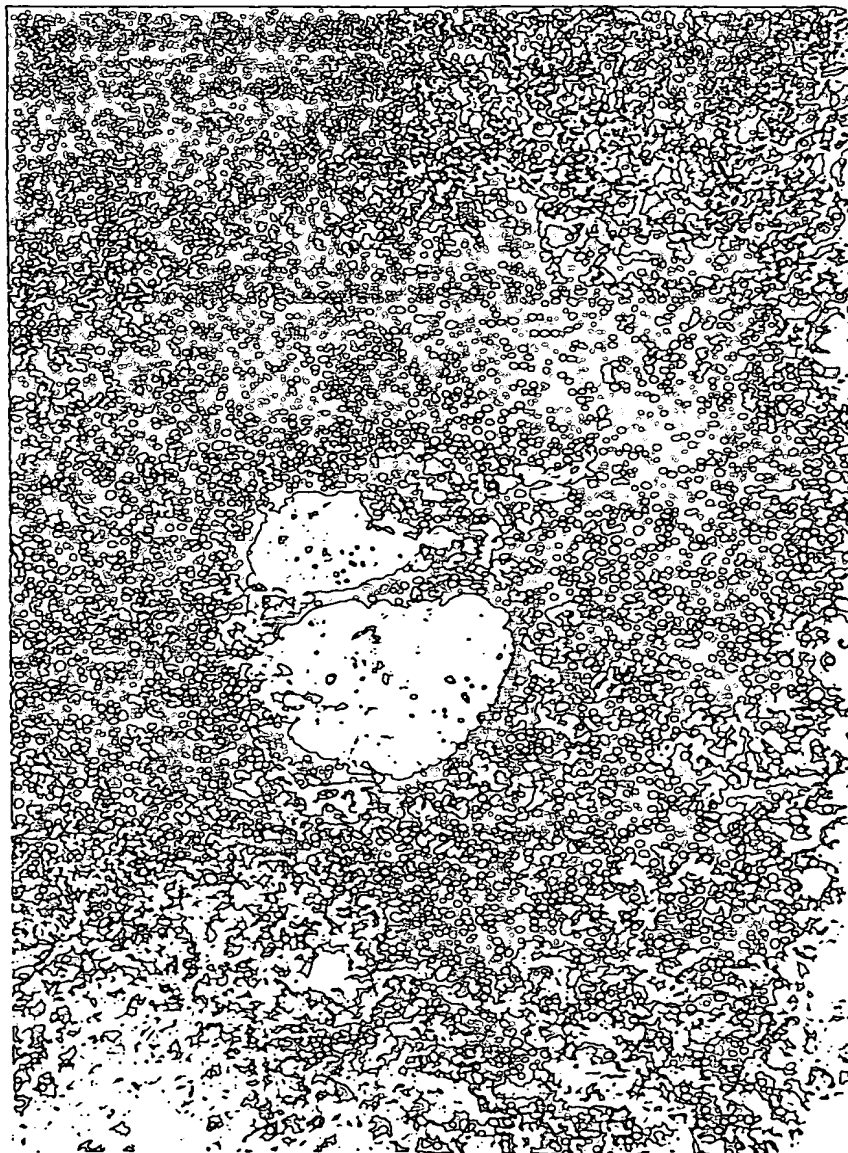
Fig. 4

FIG. 5A

A. SYNGENEIC RECONSTITUTION
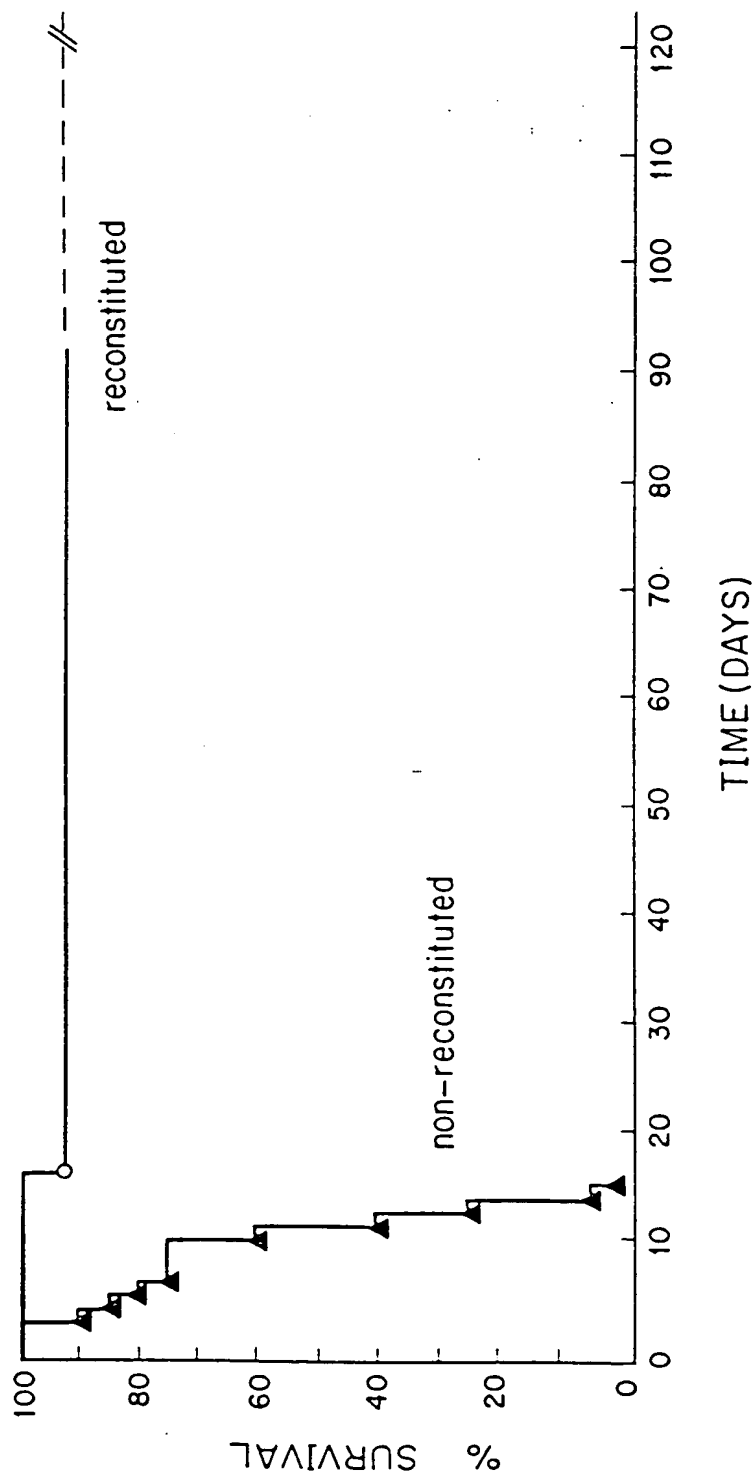
FIG. 5B

B. SEMI-ALLOGENEIC RECONSTITUTION
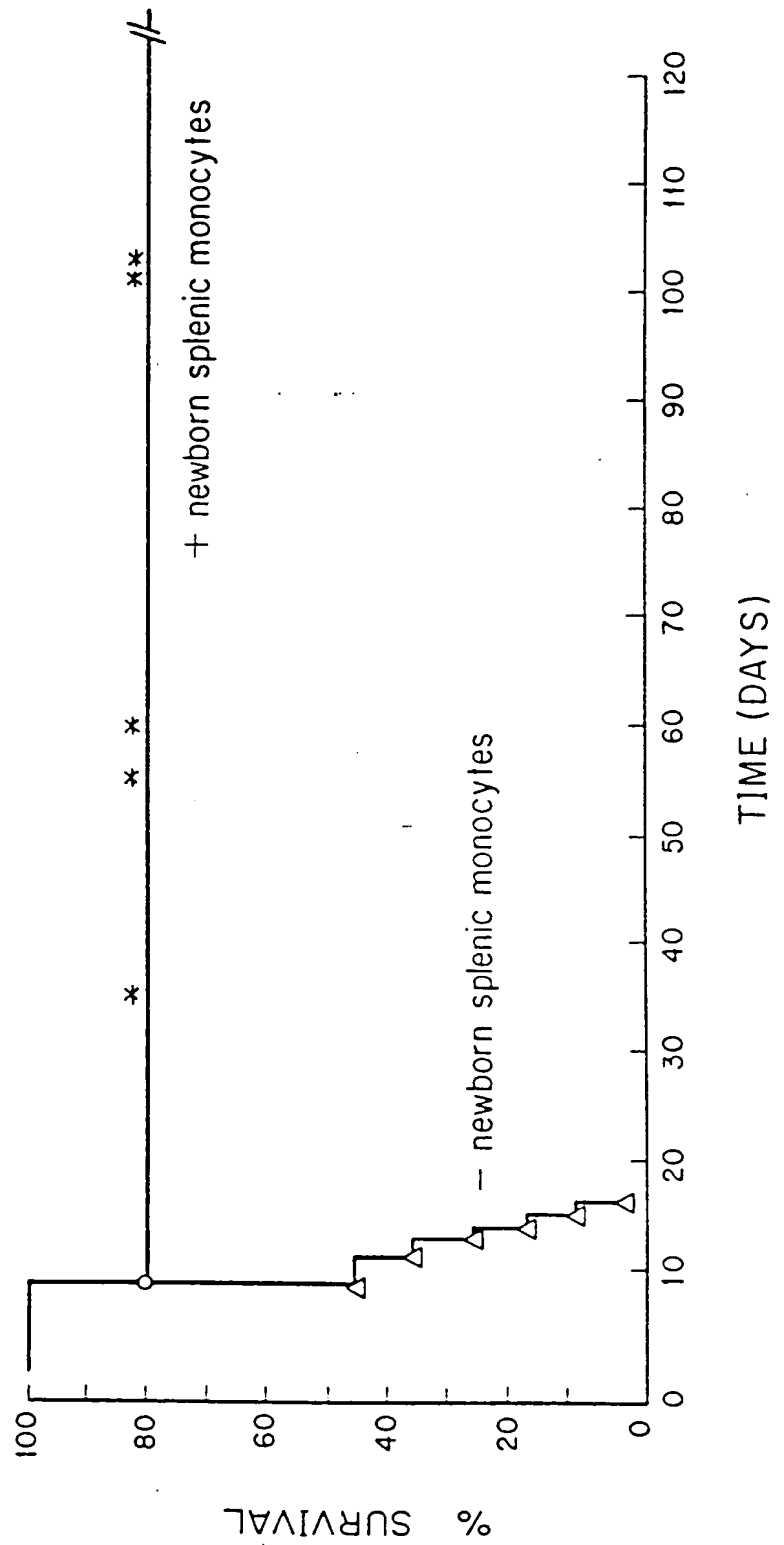
FIG. 5C

C. ALLOGENEIC RECONSTITUTION
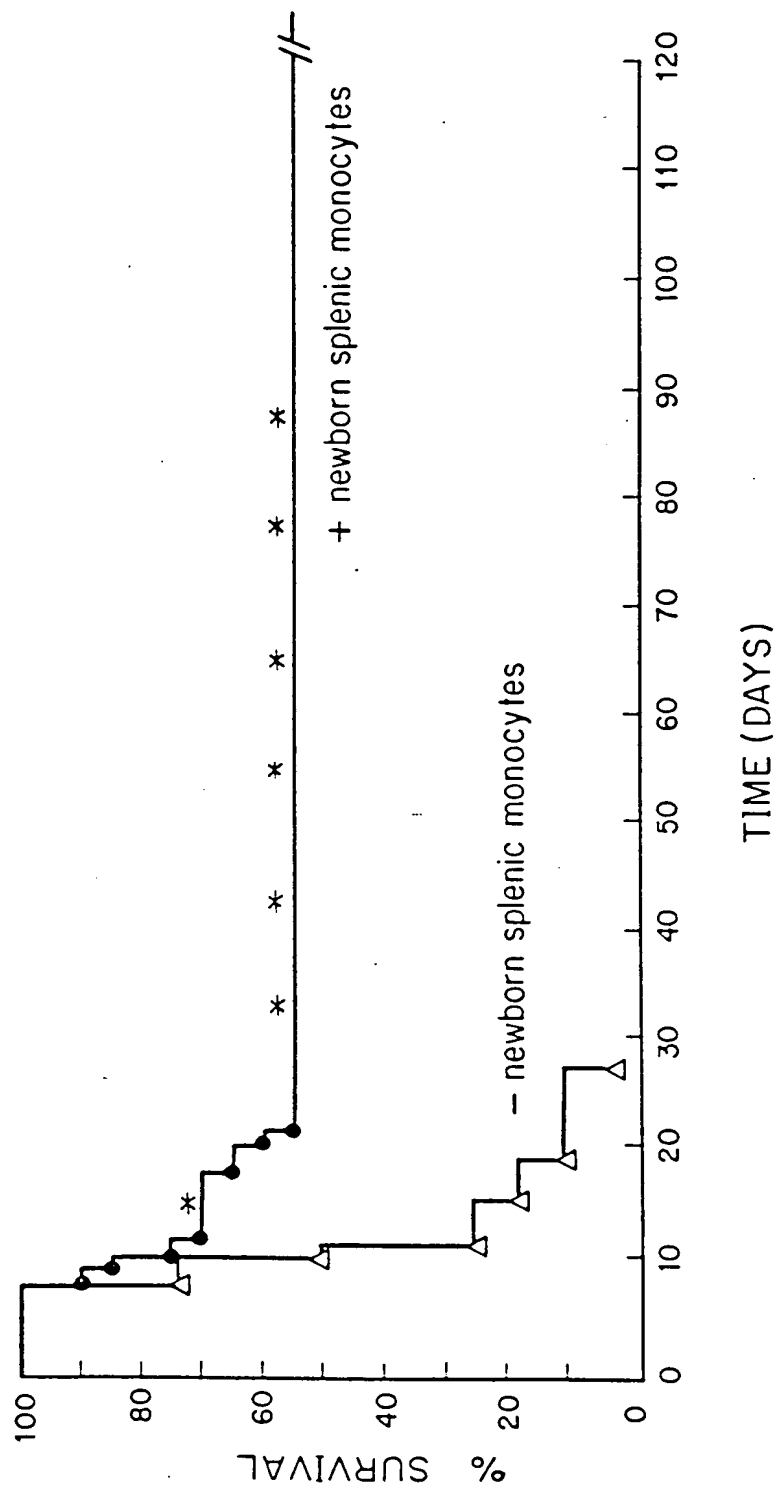
SPLEEN

Fig. 6c

LIVER

Fig. 6b

SKIN

Fig. 6a
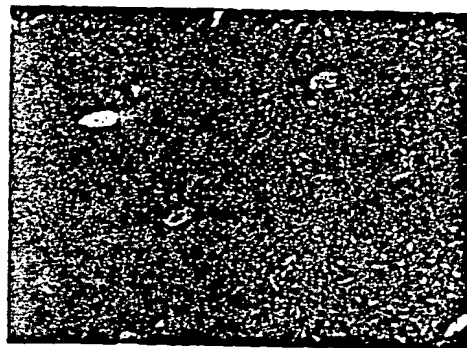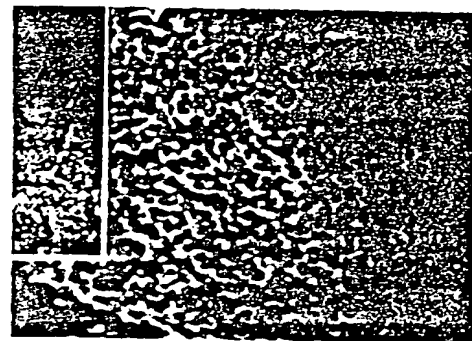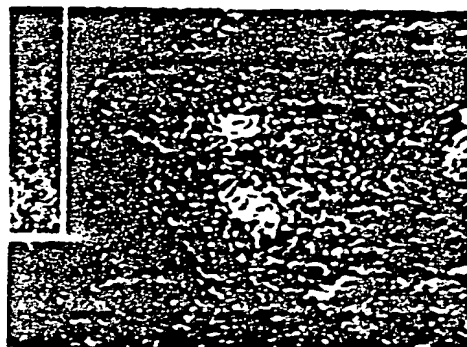
SPLEEN

Fig. 6f
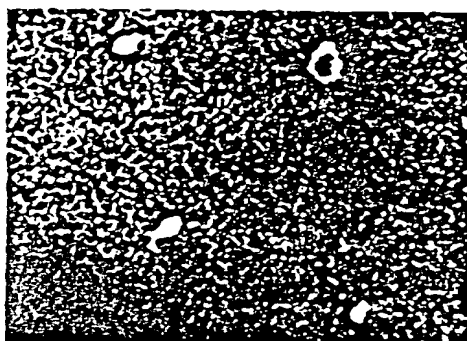
LIVER

Fig. 6e

SKIN

Fig. 6d

SPLEEN
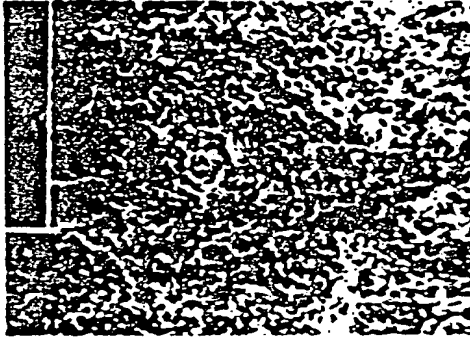
Fig. 6i

LIVER

Fig. 6h

SKIN

Fig. 6g

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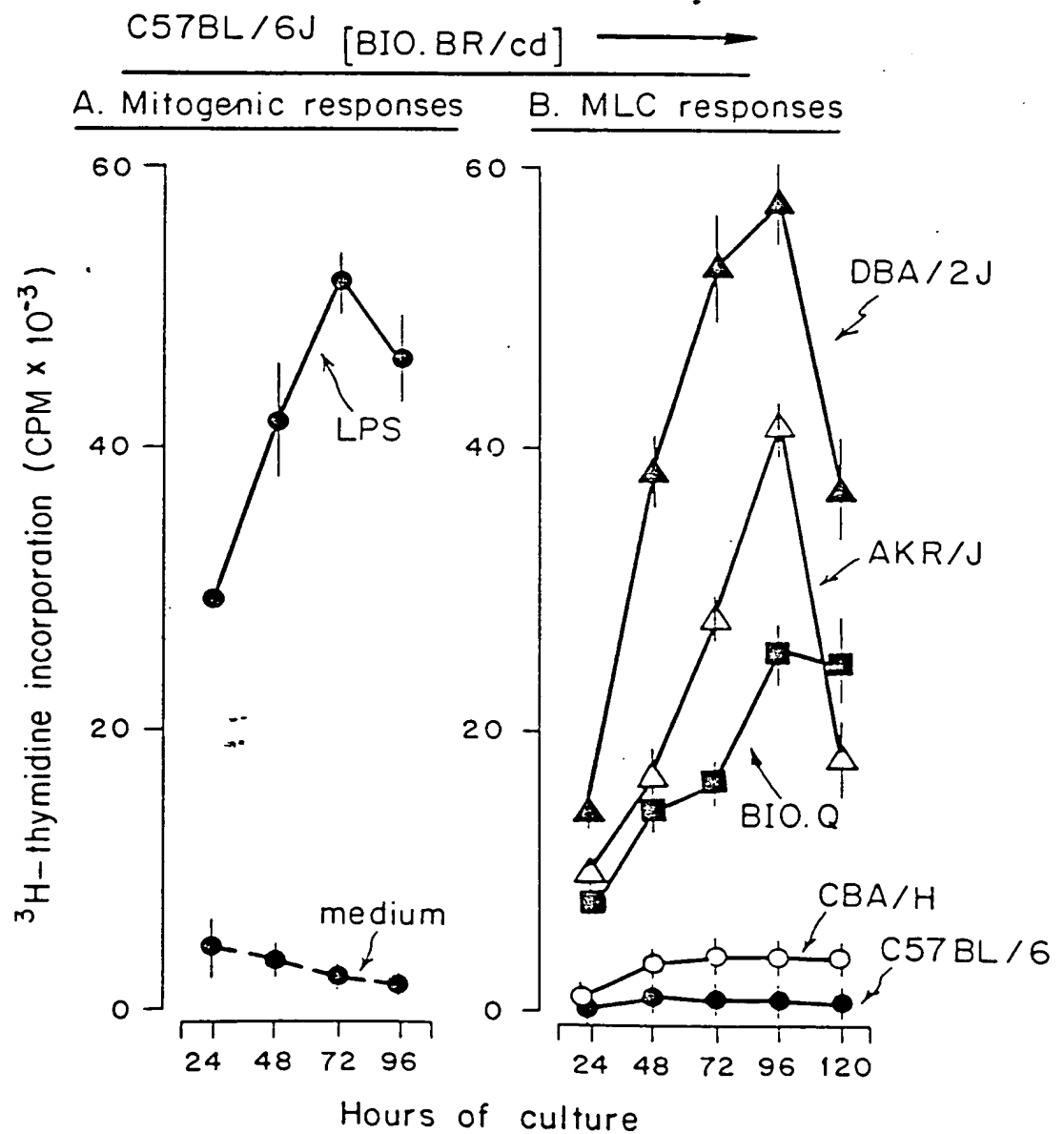
FIG. 7

§ (C57BL/10 Sn x BIO.BR/cd) F₁ [BIO.BR/cd]

A. Mitogenic responses   B. MLC responses
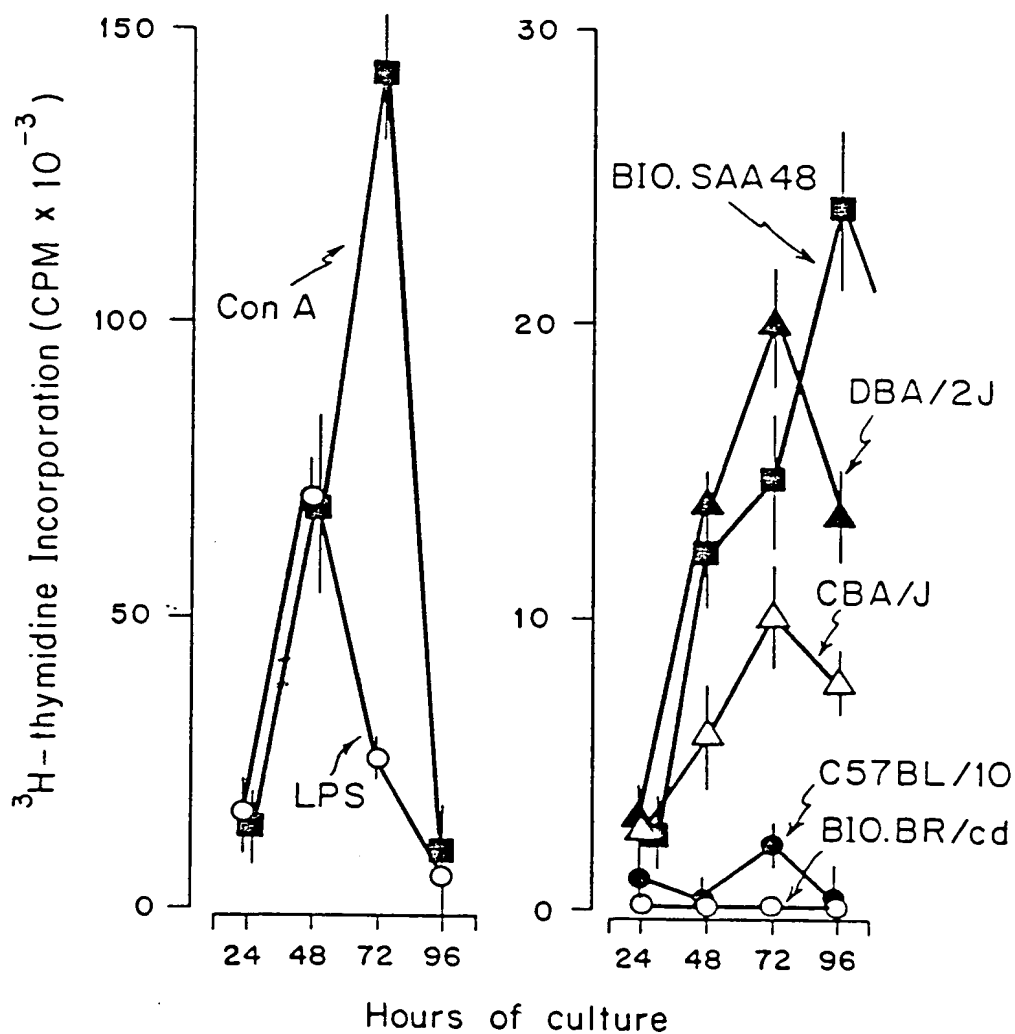
FIG. 8

C57BL/6J [BIO.BR/cd] ⟶

A. Mitogenic responses   B. MLC responses

Hours of culture

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12N 7/00; C12N 15/00; C12P 21/00
US CL : 435/172.3

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴	
Classification System ⁵	Classification Symbols
U.S.	435/68,70,91,172.3,235,317.1,320,189,195,212 935/14,32,34,56,57,60,70

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁶
CHEMICAL ABSTRACTS DATA BASE (CAS) 1967-1988 BIOLOGICAL ABSTRACTS DATA BASE (BIOSIS) 1969-1988: KEY WORDS: STEM CELL, OXALASE, URICASE, AAV, VECTOR, DEHYDROGENASE, OXIDASE

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
P,Y	Chemical Abstracts, Volume 106, No. 20, issued 25 May 1987 (Columbus, Ohio, USA), CAMPOS ET AL., "Nodule specific genes in Phaseolus vulgaris", see page 166, column 2, the abstract no. 170031k, Mol Genet. Plant-Microbe Interact, Proc. Int. Symp. 3rd, 1986, 115-117 (Eng).	1-12,17-33,35-42,53-67,78-86
Y	MOLECULAR AND CELLULAR BIOLOGY, Volume 5, issued November 1985, (Washington, D.C., U.S.A), (TRATSCHIN ET AL.), "Adeno-Associated Virus Vector for High-Frequency Integration, Expression, and Rescue of Genes in Mammalian Cells", see page 3251.	1-89

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ⁴	Date of Mailing of this International Search Report ⁸
13 June 1988	1 4 JUL 1988

International Searching Authority ¹	Signature of Authorized Officer ²⁰
ISA/US	Stephanie Seidman

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	MOLECULAR AND CELLULAR BIOLOGY, Volumn 6, issued August 1986, (Washington, D.C. U.S.A.), (TRATSCHIN ET AL.), "Negative and Positive Regulation in trans of Gene Expression from Adeno-Associated Virus Vectors in Mammalian Cells by a Viral rep Gene Product", see page 2884.	1-89
Y	JOURNAL OF VIROLOGY, Volume 51, issued August 1984, (Washington, D.C., U.S.A.), (HERMONAT ET AL), "Genetics of Adeno-Associated Virus: Isolation and Preliminary Characterization of Adeno-Associated Virus Type 2 Mutants", see page 329.	1-89
Y	NATURE, Volume 318, issued 14 November 1985, (London, England), (KELLER ET AL), "Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic prescursors", see pages 153-154.	1-89
Y	NATURE, Volume 320, issued 20 March 1986, (London, England), (HOCK ET AL), "Retrovirus-mediated transfer and expression of drug resistance genes in human haematopoietic progenitor cells", see pages 276-277.	1-89
Y	NATURE, Volume 310, issued 9 August 1984, (London, England), (WILLIAMS ET AL), "Introduction of new genetic material into pluripotent stem cells of the mouse", see pages 476 and 478-480.	1-89
P,Y	VIROLOGY, Volume 162, issued February 1988, (New York, New York), (LAFACE ET AL), "Gene Transfer into Hematopoietic Pfrogenitor Cells Mediated by an Adeno-Associated Virus Vector", see pages 483-486.	1-89